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Clinical and Molecular Genetics of Fraser Syndrome

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Abstract

Fraser syndrome (FS; MIM 219000) is a rare, heterogeneous congenital malformation disorder characterized by cryptophthalmos, syndactyly and urogenital defects. The entity is named after George Fraser who described two unrelated patients with the same congenital malformation disorder consisting of cryptophthalmos, ear anomalies, genital anomalies and syndactyly. More than 250 cases have thus far been reported in the literature.

Robin Winter had speculated that FS is the human equivalent of the murine blebbing mutants in which mutations at five loci give the same phenotype as FS. Mutations in two human genes are known to cause FS and there are three further candidate genes. *FRAS1,* the first locus identified, encodes an Extracellular Matrix (ECM) protein whose domain structure suggests a structural role within the ECM as well as in cell signalling. There are several genes similar to *FRAS1* in the human genome. These are called *FREM1-3* (Fras-Related-Extracellular-Matrix1-3). In patients with FS, missense mutations have recently been identified in a second gene, *FREM2*. The other FREM genes have not been associated with FS as yet. Mutations in *Grip1* have been identified in eye blebbing (*eb*) mutants. To date, no *GRIP1* mutations have been identified in FS patients.

Clinical data and DNA samples were collected from 59 affected individuals from 25 consanguineous and 15 non-consanguineous families. Evaluation of the clinical findings in this group revealed a higher frequency of abnormalities of the skull, larynx, umbilicus, urinary tract and anus, whereas mental retardation and cleft lip/ palate were less often observed than previously reported. Mutation analysis of the genes known or suspected to be involved in FS resulted in the identification of six *FRAS1* mutations and two *FREM2* mutations. Linkage analysis in consanguineous families indicated further evidence of linkage to *FRAS1*, *FREM2*, *FREM1* and *GRIP1*. Genotype–phenotype analysis revealed that skull ossification defects, umbilical, urinary tract, and anorectal abnormalities were more frequently reported in patients with a *FRAS1* mutation than in all other FS patients. Based on these results the existing diagnostic criteria were re-evaluated and the definition of new diagnostic criteria for FS is suggested here along with a discussion of the findings.

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Thank you Pete, for teaching me to approach science with a critical and analytic eye. I thoroughly enjoyed working in your department.

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Lastly but most importantly, I would like to thank my family, especially my husband Bart, and our sons Olivier and Julius, who have been a source of support and encouragement throughout.

"Your Fingers are your Eyes" (1954-1957)

"Is the baby perfect? What is it, a boy or girl?" The midwife turned slowly towards me, paused for a few seconds and in a very quiet voice said, "I don't know. I am going to send for the doctor because there seems to be something wrong with the baby's eyes."

The doctor arrived, examined the baby and after what seemed an eternity, the doctor finally spoke, "I'm sorry, but your baby appears to have no eyes, the ears are rather tiny, but otherwise, they seem fine. I must regrettably have the baby admitted to hospital, but there isn't very much hope of the child surviving. A child so malformed could not possibly last the night."

After ten days we had a request from the hospital to telephone them. Hen phoned the hospital to be told, "You can have your baby home if you would like to."

To Hen's immediate question, "Can you tell me then what is wrong with our baby?" the answer came back, "Not very much really. We seem to think the baby has an excess of skin in various places which can be cured by a series of operations." Hen was then assured that the baby was definitely male but no other details were given.

At one year, he was admitted to the hospital for a hernia operation. Prior to the operation, the clinic doctor had raised the question of the indeterminate sex. She was not convinced that a proper diagnosis had been made and asked if a skin section could be carried out at the same time as the operation. This method of determination was still in its infancy being a very new advance in medical science but had the greatest degree of accuracy. The outcome - the baby was a girl; hence the change of name and sex from Michael John to Michele Joan.

Three years later, I became pregnant again. I was very worried about having another handicapped baby but the doctor said, "It will never happen again." How wrong he was!

When I was eight month's pregnant, I went into premature labour and had to be rushed to hospital. This baby was born exactly the same as Michele except that it had clubbed feet and eyes something like a chicken's. (This was a nurse's description). The baby had a double eyelid. There was no urine canal or vagina so it would have been impossible to pass urine. I was told later that surgery would have been unable to rectify these defects.

I think there might be a genetic problem somewhere along the line. Nowadays we would have had genetic counselling immediately after the first child was born. Doctors did take blood samples from us as a family and did everything they could in those days. Nearly five years later, one of the doctors at the Ellen Terry Home for Blind, Mentally Handicapped children, said that it was probably because one of the genes hadn't "gone together" properly.

Fragments from the memoirs of Beryl Snelling, mother of a patient included in this study.

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List of abbreviations

AER	Apical Epidermal Ridge
bl	Blebbed
BMP	Bone Morphogenic Protein
bp	Base pair
BSA	Bovine Serum Albumin
cM	CentiMorgan
CSPG	Chondroitin Sulphate Proteo Glycan
DNA	Deoxyribo Nucleic Acid
dNTP	Deoxy Nucleoside Triphosphate
E	Embryonic day
eb	Eye blebs
ECM	Extra cellular matrix
EDTA	Ethylenediamine tetra-acetic acid
ENU	N-Ethyl-N-Nitroso Urea
ERG	Electro Retinogram
FGF	Fibroblast Growth Factor
fh	Foetal haematoma
FREM	Fras Related Extracellular Matrix
FS	Fraser syndrome
G	Acceleration due to Gravity
GRIP	Glutamate Receptor Interactive Protein
GT	Genital Tubercle
heb	Head blebs
kb	Kilobase
kPa	kilo Pascale
L	Litre
LODD	Logarithm of the Odds
LPA	Linear polyacrylamide
m	Milli
M	Molar
Mb	Megabace

μ	Micro
my	Myelencephalic blebs
n	Nano
p	Pico
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PDZ	Postsynaptic density-95/ Disc large/ Zonula occludens-1
SNP	Single Nucleotide polymorphism
TAE	Tris-acetate/ EDTA
TBE	Tris-borate/ EDTA
TE	Tris/EDTA
Tel	Telomere
TGF	Transforming Growth Factor
Tm	Melting Temperature
TM	Trans Membrane domain
UV	Ultra violet
V	Volt
VEP	Visual Evoked Potential
VWF	Von Willebrand Factor

Chapter 1

Introduction

Chapter 1 Introduction

1.1 Fraser syndrome

1.1.1 Historical Overview

Fraser syndrome (FS; MIM 219000) is a rare, heterogeneous congenital malformation disorder characterized by cryptophthalmos, syndactyly and urogenital defects. The first report of this condition dates from 1872, when Zehender introduced the term cryptophthalmos when he reported on a female child with bilateral 'hidden eyes', in combination with hypertelorism, syndactyly, umbilical hernia, abnormal genitalia, anal stenosis and a hoarse voice. Post-mortem investigations showed that she also had an encephalocele (Zehender et al., 1872). In 1883, Chiari referred to a stillborn female with cryptophthalmos and associated left renal agenesis and right renal hypoplasia (Chiari, 1883). Francois (1965) described this combination of congenital anomalies as 'malformation syndrome with cryptophthalmia' and suggested four characteristics: (1) cryptophthalmos, (2) dysencephaly comprising meningocele, cleft palate, cleft lip, ear and nasal abnormalities (3) syndactyly and (4) genital malformations. Sugar (1968) named this pattern of congenital malformations 'cryptophthalmos-syndactyly syndrome' when he reported on a patient with characteristic facial features (unilateral cryptophthalmos, unilateral symblepharon, absent brow hair, extension of temporal hair to temporal edge of left supra-orbital region) and cutaneous syndactyly of the hands (Sugar, 1968). Urogenital abnormalities were not present in this case. In 1962, Fraser described a distinctive "cryptophthalmos syndrome", in his report on two unrelated female patients with the same congenital malformation disorder consisting of cryptophthalmos, ear anomalies, genital abnormalities and syndactyly (Fraser, 1962). Ide illustrated the importance of ultrasound investigations in FS by reporting on a variety of skeletal defects that can be found in FS (Ide and Wollschlaeger, 1969). Lurie proposed that renal agenesis should also be a diagnostic feature of cryptophthalmos syndrome (Lurie and Cherstvoy, 1984). Koenig described a continuous spectrum of eye defects observed in patients with the cryptophthalmos- syndactyly syndrome, varying from mild anomalies of the eyebrow to cryptophthalmos, and suggested that the

diagnosis of cryptophthalmos- syndactyly syndrome should be considered in patients with a combination of acrofacial and urogenital abnormalities with or without cryptophthalmos (Koenig and Spranger, 1986). Since cryptophthalmos is not an obligatory feature of this syndrome, it was suggested that the eponimic designation 'Fraser Syndrome' is preferable for the cryptophthalmos-syndactyly syndrome (Meinecke, 1986).

Figure 1.1: The oldest Fraser syndrome patient and Professor Fraser.
This patient (now 51 years old) is one of the patients who Prof. Fraser described in 1962. Later the eponimic designation 'Fraser Syndrome' has been used instead of Cryptophthalmos –Syndactyly syndrome.

Thomas defined diagnostic criteria for this condition to enable a separation of patients with syndromic- and isolated cryptophthalmos (Thomas et al., 1986). He described 4 major (cryptophthalmos, syndactyly, abnormal genitalia and a sib with cryptophthalmos syndrome) and 8 minor criteria (congenital malformations of the nose, ears, larynx, cleft lip and /or palate, skeletal defects, umbilical hernia, renal agenesis, and mental retardation). He suggested that the diagnosis could be made when 2 major and 1 minor - or 1 major and 4 minor criteria are present in a patient (Table 1.1). He mentioned that "an exception was made in the case of sibs of affected individuals who were included even when they did not meet our criteria"

Major Criteria	Minor Criteria
Cryptophthalmos	Renal agenesis
Syndactyly	Laryngeal abnormalities
Genital abnormalities	Malformed ears
Affected sibling	Malformed nose
	Cleft lip/ palate
	Skeletal defects
	Umbilical hernia
	Mental retardation

Table 1.1: Diagnostic criteria for FS according to Thomas et al. (1986)

Based on these criteria, Thomas reviewed 124 cases of cryptophthalmos. Only 97 cases seemed to have cryptophthalmos as part of a congenital malformation disorder, 27 patients had isolated cryptophthalmos. Of these 97 cryptophthalmos syndrome cases, 86 fulfilled the diagnostic criteria, 11 cases remained unclassified. Since renal agenesis seemed to be present in 88% of the cases, he proposed that Fraser syndrome should be considered in the differential diagnosis of cases with multiple congenital anomalies associated with renal agenesis even in the absence of cryptophthalmos. Several reviews have been published since then (Boyd et al., 1988; Gattuso et al., 1987; Slavotinek and Tifft, 2002).

Figure 1.2: Clinical Features Fraser Syndrome

(1) neonate (2) infant (3) adult. a): Facial features comprising complete cryptophthalmos, cleft lip/ palate, broad nose with midline groove towards the tip. (b) Profile of the face with low set posteriorly rotated ears, extended temporal hair growth to temporal edge of the orbita. (c): Variable degree of syndactyly of hands and foot.

a] b]

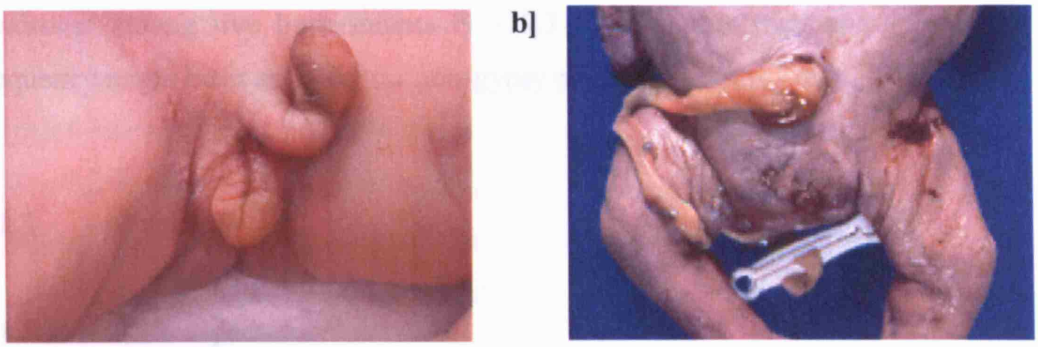


Figure 1.3: Genital abnormalities observed in FS

(a) Deformity of the foreskin; with a normally formed underlying penis (b) ambiguous genitalia in a female FS foetus

In the most recent review paper, Slavotinek re-evaluated Thomas' diagnostic criteria for 117 published cases that were diagnosed with FS or cryptophthalmos since Thomas' review (Slavotinek and Tifft, 2002). She suggests that the diagnosis should be made with caution in probands and families without cryptophthalmos, although in the presence of other typical findings, cryptophthalmos is not essential for the diagnosis. She doubts the importance of minor criteria such as orofacial clefting, umbilical hernia and mental retardation and emphasizes that gastrointestinal tract anomalies are more useful in making the diagnosis. She also found that patterns of malformations as seen in Fraser syndrome are also observed in other syndromes and associations without cryptophthalmos, suggesting that modifier genes or tri-allelic inheritance may explain the phenotypic variation in FS.

1.1.2 Incidence

Martinez-Frias reported on seven cases with Fraser syndrome identified in a series of 1,405,374 live born infants and 9,042 stillborn children surveyed by the Spanish Collaborative Study of Congenital Malformations between April 1976 and March 1997 (Martinez-Frias et al., 1994). She concluded that the minimal estimated frequency of Fraser syndrome is 0.43 per 100,000 live born infants and 11.06 per 100,000 stillbirths. Four of the seven cases she found were Gypsies for whom the frequency of this

syndrome among live born infants is 129.3 times higher than non-gypsies, so the frequency might be even lower for non-gypsy populations.

1.1.3 Prognosis

1.1.3.1 Life expectancy

About one quarter of FS cases are stillborn, another quarter dies within the first year of life (Boyd et al., 1988). The oldest patient affected with Fraser syndrome is a 51-year-old woman who is one of the original patients described by Fraser in 1962 (Fraser, 1962). (Fig. 1.1 and described in Chapter 2 as patient 38).

Life expectancy of patients with Fraser syndrome varies according to the severity of the phenotype (Karas and Respler, 1995). The prognosis of Fraser syndrome is essentially based on the severity of renal and laryngeal conditions. Respiratory failure due to laryngeal stenosis and bilateral renal agenesis is often the cause of death in neonates. Subglottic stenosis is most often first diagnosed at the time of intubation or reconstruction procedures (Ford et al., 1992). Laryngeal atresia is of most immediate concern because of the need to provide an adequate airway via either incubation or tracheostomy. Patients usually require a tracheostomy while awaiting final repair of the laryngeal defect. This is usually performed when the child is about 1-2 years old and when adequate cartilage development has occurred. However, recently a successful intra-uterine tracheal decompression was performed in a hydropic FS foetus with a congenital high airway obstruction syndrome (CHAOS), which was associated with laryngeal stenosis in FS (Kohl et al., 2006). This case will be described in Chapter 2 as patient 21).

1.1.3.2 Visual impairment

Prognosis for vision is very poor, in most cases there is no assessment of visual function, although some patients have normal VEP (Visual Evoked Potential) and ERG (electro retinogram) results and are able to distinguish light and dark and occasionally movements (Hing et al., 1990).

Surgical procedures to open eyes in complete cryptophthalmos are often associated with poor prognosis, but have been successful in a number of cases with incomplete cryptophthalmos, with results varying from a severe visual impairment to the ability to distinguish light and dark, with a visual acuity of 1/20 (Gattuso et al., 1987). More recently however, excellent long-term visual results have been reported in a patient with incomplete cryptophthalmos who underwent surgical reconstruction of her eyes (Bergwerk et al., 2004).

1.1.3.3 Reproduction

Since none of the described Fraser syndrome patients has reproduced, it is most likely that there is a considerable reduction in fertility due to the genital malformations associated with the condition. However, one of the patients, who will be described later in this thesis, underwent a correction for a vaginal atresia at early age and had regular periods between 15 and 50 years of age. However the possibility of whether this patient, may have been fertile remains unknown since she, like many patients with severe congenital malformation disorders had experienced limited reproductive opportunities, which in itself could mimic a reduction in fertility.

1.1.4 Intra familial clinical variability

There have been controversial reports of intra-familial variation of the clinical phenotype. Lambert (1989) and Slavotinek (2002) described a very similar clinical expression of the disease within the same family, with a strong phenotypic similarity observed in families of both severely affected and mildly affected. (Lambert et al., 1989; Slavotinek and Tifft, 2002). However, others described a variable expression in affected sibs which could possibly be explained by either less severe manifestations in homozygotes, or a less severe phenotype in heterozygotes (Azevedo et al., 1973; Bieber et al., 1982; Rousseau et al., 2002).

1.1.5 Prenatal diagnosis

Prenatal diagnosis of Fraser syndrome remains difficult and has been published several times. (Carlson, 1991; Comas et al., 1993; Hedrick et al., 1994; Ramsing et al., 1990; Serville et al., 1989). Both oligo- and polyhydramnios are described in FS.

Oligohydramnios caused by renal agenesis is associated with hyper echogenic lungs and flattening of the diaphragm due to the inability to excrete foetal lung liquid in to amniotic fluid. Polyhydramnios due to upper airway obstruction can lead to oesophageal compressing by the expanded lungs and subsequent impaired swallowing of amniotic fluid (Hedrick et al., 1994). Because of the inconstant sonographic findings in several reported cases of prenatally diagnosed FS, Berg proposed sonographic markers for the prenatal diagnosis of this disorder (Berg et al., 2001) (Table 1.2). There are however several pitfalls; (1) the diagnosis of syndactyly is often impaired by the associated oligohydramnios (2) laryngo-atresia can be associated with both pulmonary hyper- and hypoplasia and (3) the reliability to detect renal agenesis is limited; renal agenesis has been demonstrated at autopsy following a normal prenatal renal scan. The latter can be explained by the prenatal observation of bilateral enlarged adrenal glands being misinterpreted as kidneys (Ramsing et al., 1990). Renal agenesis can also be present despite a normal amount of amniotic fluid; polyhydramnios, due to laryngeal stenosis, can sometimes compensate for the oligohydramnios that is caused by the renal agenesis, resulting in normal amount of amniotic fluid (Ramsing et al., 1990). The diagnosis can also be missed if there is no family history of a previous affected child.

Hyperechogenic lungs
Laryngeal stenosis/ atresia
Oligohydramnios
Ascites
Renal agenesis/ dysplasia
Microphthalmia/ hypertelorism
Hydrocephaly
Syndactyly
Ear defects
Ambiguous genitalia

Table 1.2: Sonographic markers for the prenatal diagnosis of FS (Berg et al., 2001)

1.1.6 Post-mortem investigations

There are a few series reporting on post-mortem investigations of foetuses and babies with FS (Boyd et al., 1988; Lurie and Cherstvoy, 1984; Thomas et al., 1986). Laryngeal stenosis, syndactyly, and renal agenesis/ dysplasia are the most consistent findings of these investigations. These anomalies were less frequently reported anomalies in the older literature, illustrating the importance of post-mortem investigations; these features may be more frequently present than previously thought and could well have been a cause of death in many cases.

1.1.7 Pathology associated with prematurity

A patent ductus ovale and ductus arteriosis can be associated with prematurity. Some ophthalmologic symptoms (foetal chamber angle, persistent papillary membrane, absence of retinal blood vessels and prominent Bergmeister's papilla) can be explained by early gestational age of the affected foetus (Brownstein et al., 1976). Empty scrotal sacs can also be normal for the gestational age (Hambire et al., 2003).

1.1.8 Oligohydramnios sequence

Many of the features observed in FS can be secondary to the renal defects. Bilateral renal agenesis is the most common cause of the renal non-function (Potter) syndrome. This oligohydramnios sequence consists of a typical facial appearance, pulmonary hypoplasia, limb positioning defects and growth deficiency (Codere et al., 1981). The face is characterized by a combination of a broad beak-like nose, distorted low set ears, and micrognathia with a marked depression between the lower lip and tip of the chin. The limb positioning defects comprise clubfeet and arthtrogryposis (Brownstein et al., 1976). If renal agenesis is present in FS, a distinction should be made between dysmorphic facial features that are characteristic for FS and the typical Potter facies caused by the oligohydranmios as a result of renal agenesis.

1.1.9 Cytogenetic abnormalities

The only cytogenetic abnormality reported so far is a balanced inversion of chromosome 9 (46,XX,inv(9)(p11q21) in a girl with FS (Schauer et al., 1990). The

phenotypically normal father and an affected foetus in the second pregnancy had the same karyotype.

1.1.10 Oncogenesis

Development of tumours is rare in Fraser syndrome. There has thus far only been one report of a FS patient who developed a gonadoblastoma in dysgenetic ovary (Greenberg et al., 1986). Gonadoblastoma arising from a dysgenetic gonad normally occur in mixed gonadal dysgenesis with Y chromosomal mosaicism (45, X/ 46,XY). The reported patient did not however show evidence of cytogenetic abnormalities.

1.2 Mouse mutants as models for human disease

Mice are the most widely used animal models of human diseases because of their small size, short life span and generation time. Their relatively cheap and easy breeding has enabled the study of genetic crosses and systematic mutagenesis programmes for decades, and many mutant phenotypes have been described (Wynshaw-Boris, 1996). Apart from arising spontaneously, mutations can be generated artificially by exposure to mutagenic chemicals (ENU) or high doses of X-rays. However, chemically and radiation induced mutagenesis has the disadvantage of being random. More recent techniques have allowed the creation of animal disease models resembling specific human diseases by introducing mutations into a particular gene in vivo. Mice can be generated with an alteration in a chosen target gene or by disrupting a particular gene with a large insertional cassette, thereby generating null-mutations (gene knock-outs) (Gordon and Ruddle, 1983). These techniques are largely used to model loss of function mutations. Homologous human and mouse mutants often show a similar phenotype, facilitating the identification of human genes. Almost all human genes have an easily identifiable mouse homologue because of the high level of conservation between human and mouse coding sequences. Once a disease gene in one species has been identified, it is possible to predict the location of that gene in other species (htpp://www.ensembl.org, htpp://www.ncbi.nlm.nih.gov/Omim/homology).

1.2.1 Mouse blebbing Mutants

There are five 'blebbing' mutants described in the mouse (Green, 1989). This blebbing mutant family comprises four mapped and one unmapped loci, known as myelencephalic blebs (*my*), blebbed blebs (*bl*), eye blebs (*eb*), head blebs (*heb*), and foetal haematoma (*fh*). Mouse blebbing mutants are characterized by uni and bi-lateral cryptophthalmos, soft tissue- and bony syndactyly and a range of other defects, comprising renal- skin-, hair- and central nervous system anomalies (Table 1.3 and Figure 1.4). Because of their extensive phenotypic overlap, Robin Winter postulated that these blebbing mutants could be a model for Fraser syndrome (Winter, 1990). They all show the characteristic prenatal appearance of sub epidermal fluid filled blebs or blisters appearing around 12 days of gestation (equivalent to approximately 37 days post fertilization in humans), covering the extremities, eyes and forebrain. These blisters enlarge, become haemorrhagic and disappear during late gestation.

Blebbing mutant	***bl***	***my***	***eb***	***hb***	***fh***
Mouse chromosomal location	**5**	**3**	**10**	**4**	**?**
Homologous region in human	**4q**	**13q**	**12**	**9**	**?**
Cryptophthalmos	+	+	+	+	+
Partial lens detachment			+		
Folded irises			+		
Anophthalmos		+			
Acrania	-	+			
Meroanencephaly	-	+			
Neural tube defects			+		
Syndactyly	+	+	+		+
Polydactyly	-	+		+	
Clubbed feet		+	+		
Renal agenesis	+	+	+	+	
Polycystic kidneys	+	+	+		
Hydronephrosis		+			

Table 1.3: Clinical features of the blebbing mutants

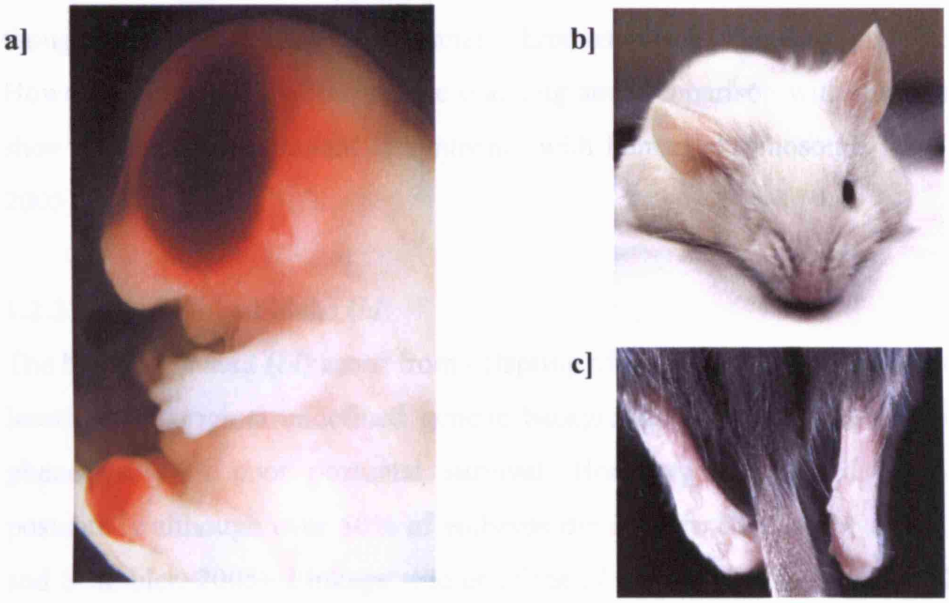


Figure 1.4: Blebbing mutants

(a) E14.5: showing hemorrhagic blisters covering eyes and toes (b) unilateral cryptophthalmos, (c) syndactyly

1.2.2 Myelencephalic blebs (*my*)

The oldest and best characterized of the 'blebbing' mutants are *the myelencephalic blebs (my)* which were discovered by Little and Bagg, following an X-irradiation experiment (Little and Bagg, 1923), although the mutation might already have been present in the original stock of experimental mice (Gruneberg, 1952). The term myelencephalic blebs originates from the first interpretation that the anomalies were the result of the overproduction of cerebrospinal fluid, leaving the myelencephalon in the forms of blebs. The myelencephalic blebbing (*my*) mutant is characterized by anophthalmos, abnormalities of the skin and hair, clubbing of the feet, a variable degree of skin syndactyly, together with occasional pre-axial polydactyly of the hindfeet, acrania, meroanencephaly, uni- or bilateral renal agenesis and more rarely polycystic kidneys or hydronephrosis (Center and Polizotto, 1992; Darling and Gossler, 1994;). The malformations are variable and frequently asymmetric. Heart defects have been described by Timmer at al. but these have not been confirmed by others (Timmer et al., 2005; Jadeja, personal communication). Homozygous *my* have good survival rates. Darling identified a second *my* allele (my^{ucl}) during the creation of an unrelated

transgenic strain, that mapped to mouse chromosome 3, in an area that was initially thought to be synthenic with human chromosome 4q (Darling and Gossler, 1994). However, refinement of the mouse mapping and comparison with the human sequence showed that the *my* mutant is synthenic with human chromosome 13q (Jadeja et al., 2005).

1.2.3 Blebbed blebs (*bl*)

The blebbed mutant (*bl*) arose from offspring of a male irradiated with neutrons, and at least on the original undefined genetic background, appears to have the most severe phenotype with poor postnatal survival. Homozygous blebbed blebs usually die postnatally although over 50% of embryos die in utero (McGregor et al., 2003; Smyth and Scambler, 2005). Linkage studies of the *bl* mutant map the gene to chromosome 5 in an area showing conversation of syntheny with human chromosome 4q. The phenotype has not been well documented; *bl/bl* mice have cryptophthalmos, distal limb defects (syndactyly), and renal agenesis. Cystic kidneys are less frequently observed, and craniofacial-blebs and polydactyly of the hind limbs that can be present in *my* mutants are not observed in the *bl* mutants.

1.2.4 Eye blebs (*eb*)

The eye blebs (*eb*) mutant arose spontaneously in a non-inbred strain of hairless mice They are characterised by disrupted eye development (partial lens detachment and folded irises), syndactyly, clubbed feet and neural tube defects (Swiergiel et al., 2000). They are reported to have the most severe renal abnormalities; apart from renal agenesis these mice can also have cystic kidneys. *Eb* is mapped at mouse chromosome 10, in an area that shows conversation of syntheny with human chromosome 12.

1.2.5 Head blebs (*heb*)

Head blebs (*heb*) is a spontaneous mutation, characterized by cryptophthalmos and sometimes polydactyly. The blebs in *heb* are more restricted to the head of the mouse, and renal abnormalities as reported in the other blebbing mutants were not reported in

heb (Varnum and Fox, 1981). *Heb* is mapped at mouse chromosome 4; equivalent to the human chromosome 9p22.

1.2.6 Foetal haematoma (fh)

Foetal haematoma (*fh*) is characterized by eye and limb abnormalities. Little is known about this mutant and it has not been mapped (Center, 1977).

1.3 Pathogenesis

It has been postulated that the bleb formation is due to epidermal fragility on the basis that head, back and tail make physical contact with the uterine wall causing the observed limb and eye defects through mechanical interference with the development of these structures (Carter et al., 1979). The asymmetrical nature of the lesions and inconsistency of number of digits involved in the syndactyly support this idea. Renal agenesis and meroanencephaly can not however be explained by this mechanism (Darling and Gossler, 1994). A defect in programmed cell death or apoptosis, is another suggested underlying mechanism for the features observed in Fraser syndrome (Thomas et al., 1986). Apoptosis in developing systems is an important process for eliminating regressing tissue regions and cells during embryonic development. It is not merely a degenerative process but rather an active and controlled phenomenon. For the formation of various structures during embryogenesis, apoptosis occurs according to precise temporal sequences and spatial patterns, and is considered to play a key role by eliminating unnecessary cells to achieve complex histogenesis and organogenesis (Sasaki et al., 2004). Apoptosis is associated with down- or up regulation of various developmental regulatory genes (Keranen et al., 1999). All cells undergo apoptosis unless rescued by survival factors (Steller, 1995). Most malformations occur in areas that are temporarily fused in utero including eyelids, digits and vagina. Separation of these structures occurs via apoptosis and failure of canalisation could lead to fused eyelids, digits, and vaginal atresia, but also the narrowness of the external auditory canal and laryngeal stenosis can be explained by failure of canalisation (Bialer and Wilson, 1988; Hambire et al., 2003; Moore and Persaud, 2003). This could be due to overexpression of survival/ anti-apoptotic factors. The same hypothesis had been

suggested as an explanation for the co-occurrence of obstructed/ narrow ducts in many different organs (external auditory canals, larynx, lacrimal ducts, vasa deferentia) in a male patient with syndactyly, microdontia, and macrocephaly (Hennekam and Cohen, Jr., 1995). In FS however, there have been no significant changes in the number of apoptotic cells in the interdigital spaces of Fras1-/- limbs observed, indicating that defective apoptosis is not the primary cause of these fusions (Vrontou et al., 2003). Others suggested that the combination of defective neural crest cell migration, proliferation, differentiation and deficient pre-programmed cell death could be a common denominator to otic, ocular, facial and laryngeal abnormalities in FS (Ramsing et al., 1990).

1.3.1 Eye development

Eye development begins at the fourth week of gestation with the formation of the optic sulcus. This soon deepens to form a hollow optic vesicle that projects laterally from the forebrain. By making contact with the surface ectoderm, the optic vesicle induces the development of the lens placode (the primordium of the lens). The optic vesicle invaginates and forms the optic cup, invagination of the lens placode leads to the formation of the lens pit. The retina forms the two layers of the optic cup.

Retinas, optic nerve fibres, muscles of the iris and cilliary body derive from the neuroectoderm of the forebrain. The surface ectoderm gives rise to lens, epithelium of the lacrimal glands, eyelids, conjunctive and cornea. Mesoderm gives rise to eye muscles (except those of the iris) and all connective and vascular tissue of cornea, iris, cilliary body, choroids, and sclera (Moore and Persaud, 2003).

1.3.1.1 Cryptophthalmos

In the second month of gestation two surface ectodermal folds, containing cores of mesenchyme, move together over the developing eye by differential growth. They contact each other around week 10 and become fused together by an epithelial seal. While the eyelids are adherent, a closed conjunctival sac exists anterior to the cornea. When the eyelids open, the conjunctiva covers the 'white' of the eye and lines the inner surface of the eyelids. The fused lids separate between around week 26 (Moore and

Persaud, 2003). Failure in the development of the eyelids results in cryptophthalmos. Complete cryptophthalmos involves covering of the eyes with skin, covering the orbits and connecting them to the underlying globe so that ocular movements can be seen beneath it. Sometimes a horizontal scar can be observed where the palpebral fissure normally lies. There is usually a varying absence of eyelashes, eyebrows, and eye defects; the eye remains underdeveloped and sightless (Waring and Shields, 1975). The pathogenesis of cryptophthalmos is still unclear. Four possibilities have been postulated: (1) Primary failure of mesodermal and ectodermal differentiation, resulting in absent eyelid folds (2) intrauterine inflammation, producing fusion of the eyelids to the globe (3) amniotic bands with pressure on the developing eyelids causing colobomas, and (4) normal eyelid fold development with mal-differentiation of the conjunctiva resulting in symblepharon (Duke-Elder, 1963; Francois, 1969). The presence of eyelashes and eyebrows favours the last hypothesis of secondary disruption rather than primary failure of eyelid fold formation (Goldhammer and Smith, 1975; Koenig and Spranger, 1986; Mena et al., 1991; Pankau et al., 1994). The third option however, could be comparable with the intrauterine bleb-formation as observed in the blebbing mutants that results in a similar ocular phenotype.

Although histo-pathological studies of cryptophthalmos revealed anterior segment dysgenesis with normal posterior parts, retinal folds have also been observed (Pe'er et al., 1987).

1.3.1.2 Anophthalmos

Anophthalmos is complete absence of the eye and can be divided in two groups: (1) primary anophthalmos, when eye development arrests early due to a failure in the formation of the optic vesicle in the fourth week of gestation and (2) secondary anophthalmos, comprising a suppression of the development of the entire forebrain; absence of the eyes is one of several anomalies.

1.3.1.3 Microphthalmos

Microphthalmos comprises a small to vestigial eyeball that can be accompanied by variable hypoplasia of the eyelids, lacrimal ducts and orbits.

Severe microphthalmos results from arrested development of the eye before or shortly after the formation of the optic vesicle in the fourth week: the eye is essentially undeveloped and the lens is absent. The eye can be larger, but associated with gross ocular defects, when eye development arrests before the retinal fissure closes in the sixth week of gestation. Interference with eye development in the eighth week or during the early foetal period results in simple microphthalmos (a small eye with minor ocular abnormalities). Mutations in PAX6, CHX10, RAX and SOX2 have been reported in patients with microphthalmia/ anophthalmia (Bar-Yosef et al., 2004; Fantes et al., 2003; Glaser et al., 1994; Voronina et al., 2004). Both anophthalmia and microphthalmia have been described in Fraser syndrome.

1.3.2 Urinary tract development

Kidney development starts with the formation of three excretory organs (the pro-, meso- and metanephros), which derive from the intermediate mesoderm. The pronephros develops in the third week of gestation and disappears around day 25. The mesonephros begins to develop before the pronephros has disappeared (day 24) and the metanephros forms before the mesonephros has entirely regressed in the fifth week of gestation. Although pronephros and mesonephros are transient organs, they both are essential to the formation of the definite kidney from the metanephros (Woolf and Jenkins, 2006). At day 28 of gestation, the mesonephric (Wolffian) duct drains into the urogenital sinus, which is formed by a division of the cloaca into the sinus and rectum by caudal extension of the urorectal septum. The ureteric bud is formed as an outgrowth of the mesonephric duct close to its entrance into the urogenital sinus. The bud penetrates the distal part of the intermediate mesoderm, rescuing it from an apoptotic fate, which then becomes metanephic mesenchyme. Due to reciprocal interactions between these two tissues, the process of nephrogenesis starts. This involves extensive branching of the ureteric bud and epithelial mesenchymal transition of the condensed mesenchyme surrounding the tips of the branching bud. This process results in the formation of the nephrons, renal pelvis, and calices.

The lower urinary tract begins to form when the initial steps of metanephric development occur. At day 37 of gestation, the ureteric bud's origin enters the bladder

to become the ureteric orifice. Between 37 and 47 days, a membrane temporarily blocks the uretero-vesical junction and the ureter becomes occluded. Subsequent 're-canalisation' of the elongating ureter is completed by 8 weeks of gestation. The bladder appears at ten weeks of gestation as a cylinder of epithelium surrounded by mesenchymal tissue that will form the smooth muscle layers of the detrusor from 13-20 weeks of gestation.

Renal agenesis is the most commonly observed renal abnormality in Fraser syndrome and is the result of either early degeneration of the ureteric bud or failure of metanephric differentiation and survival. When the ureteric bud fails to reach and/ or induce the metanephric mesenchyme, the mesenchyme becomes apoptotic. A variety of growth factors (BMP7, FGF7, GDNF, cRET) and transcription factors (LIM1, PAX2) modulate ureteric bud branching and keep bud development in step with that of other tissue types (Woolf and Jenkins, 2006). Proteoglycans of the extra cellular matrix are also required in this process (Davies and Fisher, 2002).

1.3.3 Limb development defects

The limb develops from the embryonic limb bud through a rapid cell proliferation process. Limb buds appear towards the end of the fourth week as slight elevations of the ventrolateral body wall. The upper limb buds develop about two days before the lower limb buds, and tissues of the limb buds are derived from two main sources, mesoderm and ectoderm (Moore and Persaud, 2003). Limb development is mediated by a number of reciprocal epithelial-mesenchymal interactions between the thickened apical epidermal ridge (AER) at the distal tip of the limb bud, and the underlying mesenchyme. Limb buds elongate by proliferation of the mesenchyme within them. During the late phase of limb morphogenesis, rays of condensing mesenchyme appear in the distal region of the bud (autopod); they grow and differentiate into cartilage (digits), whereas interposed tissues (interdigits) undergo programmed cell death. Digit formation is thus a combined result of chondrogenic outgrowth and interdigital cell death. The underlying mechanism remains largely unknown; a number of bone morphogenetic proteins (BMP2, 4, and 7) and Fbn2 have been implicated in specifying the chondrogenic and apoptotic fate and in regulating the digit identity (Arteaga-Solis et

al., 2001; Hogan, 1996). There is also increasing evidence for the involvement of extra cellular modulators such as proteoglycans and tissue proteases in modulating the control of this specific patterning (Christian, 2000).

1.3.4 Genital anomalies

Epithelial-mesenchymal interactions play an essential role in regulating a wide variety of developmental processes and so they do in genital development. The external genital anlage, GT (Gential Tubercle) differentiates in a penis and clitoris in male and females respectively. GT development may have some similarities to limb appendage development with both structures exhibiting organ outgrowth (Yamada, 2005). There is evidence that some BMPs could negatively affect the proximo-distally orientated outgrowth of the GT with regulatory functions on cell proliferation and apoptosis (Suzuki et al., 2003).

1.4 Molecular Genetics

1.4.1 Linkage analysis

Genetic linkage occurs when alleles at genetic loci are passed on together to the next generation, with a greater probability than could be explained by chance. The closer loci are on a chromosome, the more likely it is that alleles of those loci will be inherited together, whereas loci that lie further apart on a chromosome are more likely to be separated by a recombination event.

Various attempts have been made to identify the 'Fraser Syndrome genes'. Linkage studies in the mouse initially revealed that both the myelencephalic blebs *(my)* and blebbed blebs *(bl)* genes mapped to mouse chromosome 5, showing homology with human chromosome 4.

Aideen McInerney, focussed on chromosome 4 and found a stretch of homozygosity in one family for 5 markers, spaced approximately 47cM apart. The highest two-point LOD score was 1.468, which could be considered suggestive of linkage if the condition was heterogeneous.

Professor Robin Winter requested a genome wide screen for six families. Research Genetics tested 50 markers spread over chromosomes 1-7, 20 and 22 and found no evidence for linkage; however they only tested one marker for chromosome 4. Homozygosity for this marker (D4S1647) was demonstrated in two families.

Professor Bob Mueller performed a second haplotype analysis in a large multiple consanguineous family with two affected patients. Although only one of the two affected patients showed homozygosity over the same region that had already been highlighted by McInerney, the other patient displayed a common haplotype for only four markers covering a distance of approximately 20 cM. So it was hypothesized that there must have been two crossover events within the ancestral chromosomes and that therefore the homozygous region should be very small.

Finally, McGregor et al. identified the first locus for Fraser syndrome on chromosome 4q21 by demonstrating linkage to *FRAS1* in ten of the twenty-two families that she included in her study. These mapping data supported mapping in the *blebbed* mutant. Mutations were subsequently identified in the *FRAS1* gene, confirming that this was the first FS gene (McGregor et al., 2003).

FRAS1 encodes a large transmembrane extra cellular matrix protein. There are three genes similar to *FRAS1* in the human genome and these are called *FREM* (Fras-related-extracellular-matrix 1-3).

1.4.2 Extra cellular matrix proteins

FRAS/FREM proteins have sequence similarity to ECM3, a component of extracellular matrix fibres that undergo dynamic reorganization during sea urchin gastrulation (Hodor et al., 2000).

The Extra Cellular Matrix (ECM) is the critical mediator of intercellular interactions that control both tissue development and structural integrity. ECM molecules are important for cell adhesion and migration, for basement membrane integrity and epithelial-mesenchymal interactions during development.

FRAS/ FREM proteins have a chondroitin sulphate proteoglycan domain (CSPG) with sequence similarity to NG2, and CalXß domains. The NG2-like domains have high affinity binding sites for basic fibroblast growth factor (bFGF) and platelet derived

growth factor-AA (PDGF-AA) that are ubiquitous growth factors with profound effects on proliferation, differentiation and survival of cells from different tissues (Hodor et al., 2000). The CSPG domains in NG2 have also been shown to interact with Col V and Col VI, which form an integral part of the cell matrix (Burg et al., 1996). The binding of collagen, bFGF and PDGF-AA can be communicated to the cytoskeleton, affecting cell movement and shape (Goretzki et al., 1999).

CalXß domains are seen in a variety of calcium transporter proteins and in other cell adhesions molecules. They contain two tandem high-affinity Ca2+ binding sites (Schwarz and Benzer, 1997). Little else is known about the CalXß domain, though normally CalXß is found in proteins that span the cell membrane and are involved in Na-Ca exchange. The CSPG repeats in the NG2-like domain of FREM2 are related to cadherin domains (Staub et al., 2002). Cadherins mediate Ca2+-dependent cell-cell adhesion and the binding of Ca2+ to cadherins is known to induce major conformational changes such as rod formation, necessary for cell adhesion and migration.

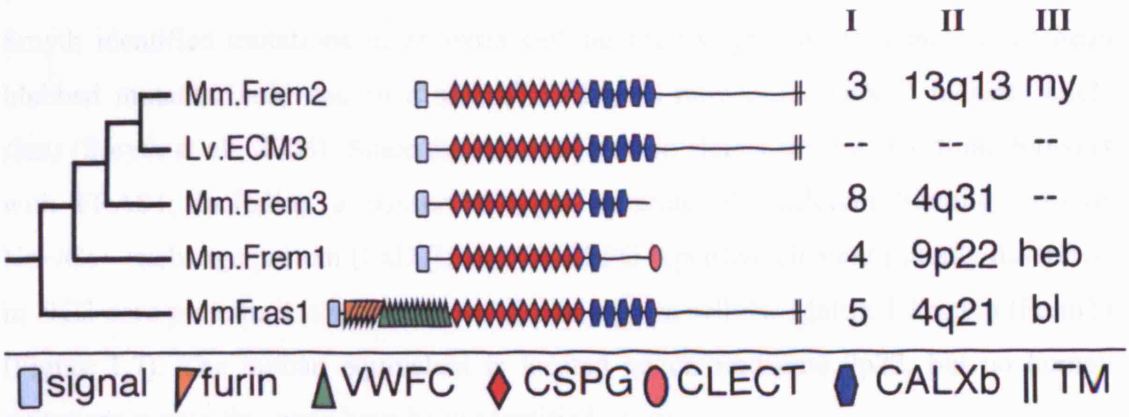


Figure 1.5: Extra cellular matrix proteins sharing the CSPG and CalXß domains (I: chromosomal location in mice II: chromosomal location in human. III: blebbing mutant (Smyth and Scambler, 2005).

1.4.3 Blebbed mutants and Fras1

Fras1 mutations were found in both the blebbed (*bl*) mutant and five unrelated Fraser syndrome patients, confirming that Fraser syndrome is a human equivalent of the murine blebbing mutants (McGregor et al., 2003). A targeted null mutation of *Fras1* has

the *bl* phenotype (Vrontou et al., 2003). FRAS1 comprises the C domain of the von Willebrand Factor, the cystein rich domain of the furin proteases (overlapped by chorin/-kielin–like domains), the NG2-like domain (comprising multiple repeats of a (CSPG) element), and a calcium-binding loop of the Na+/Ca+ exchange proteins (CalXβ). Mutations identified in *Fras1* in both Fraser syndrome patients and blebbed mutants led to a premature truncation of the protein (Figure 1.6).

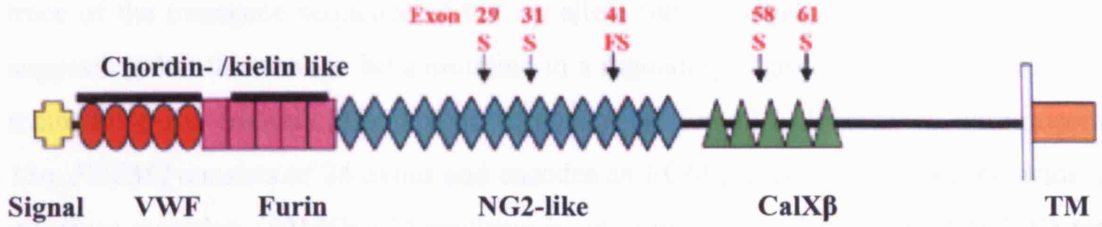


Figure 1.6: FRAS1 protein (Arrows indicate the mutations detected in Fraser syndrome patients (McGregor et al., 2003)

1.4.4 Head blebbed and Frem1.

Smyth identified mutations in an extra cellular matrix gene in both the classic head blebbed mutants (*heb)* and in a second N-ethyl-N-nitrosourea (ENU) induced allele *(bat*) (Smyth et al., 2004). Since the encoded protein shares several structural domains with FRAS1, including a conserved signal peptide, the calcium binding loop of Na+/Ca+ exchange protein (CalXβ) and the CSPG repetitive element initially described in NG2 core protein, it is termed Fras1 Related Extra cellular Matrix 1 Protein (Frem1) (Figure 1.7). The human equivalent is located on chromosome 9p22, but no human mutations within this gene have been identified as yet.

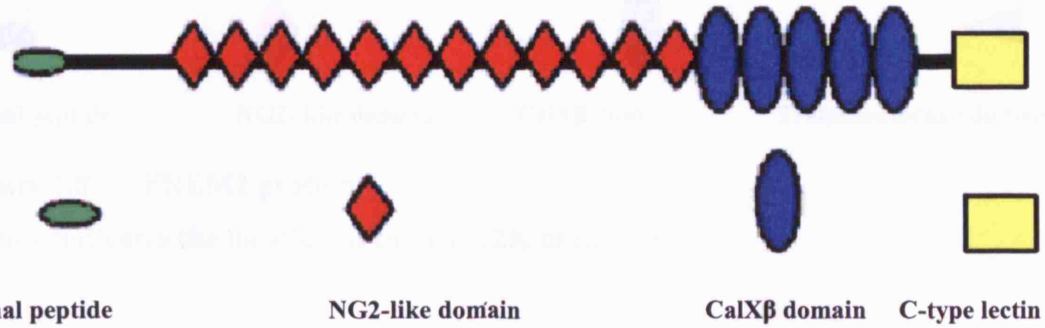


Figure 1.7: FREM1 protein

1.4.5 Myelencephalic blebs and FREM2

Jadeja mapped the *my* blebbed strain to *Frem2*, a gene related to *Fras1* and *Frem1*, on chromosome 3 (Jadeja et al., 2005). *Frem2* has greater identity to ECM3 than FRAS1 (53 versus 30% identity). It also has a transmembrane domain, however, it lacks the N-terminal (chordin-/ kielin-like) domains of FRAS1 (Figure 1.8). Complementation analysis showed that a gene- trap mutation of *Frem2* was allelic to *my* mutation. No trace of the transgene sequence of the *my* allele (*my* ^{ucl}) could however be identified, suggesting that there might be a mutation in a regulatory region or in coding exons not included in the analysis. The human equivalent of *Frem2* is located on chromosome 13q. *FREM2* consists of 24 exons and encodes an ECM protein of 3091 amino acids. A missense mutation (5914G→A) resulting in an amino acid substitution (E1972K) was identified in the CalXβ domain of this gene, in two unrelated patients with Fraser syndrome (Jadeja et al., 2005). Protein structure analysis of FREM2 carried out by Martin Taylor suggests that FREM2 binds three Ca2+ ions. The E1972K residue is involved in the binding of at least one of these ions, the change in charge from an acidic to a basic amino acid could abolish the binding of at least one, if not all three calcium ions. This is likely to have a substantial effect on the structure of FREM2 and may affect protein-protein interactions.

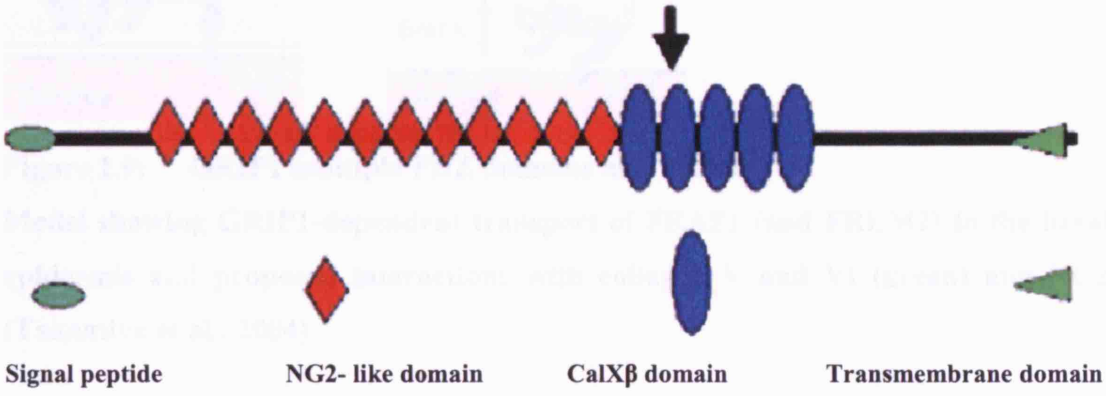


Figure 1.8: FREM2 protein

Arrow indicates the location of the E1972K mutation.

1.4.6 Eye blebs and Grip1

Takamiya identified that mutations in *Grip1* (Glutamate Receptor Interacting Protein1) cause the eye blebs mutant *(eb)* (Takamiya et al., 2004). GRIP1 belongs to a small family of cytoplasmic proteins containing 7 PDZ (Postsynaptic density-95/Discs large/Zone occludens-1) domains. PDZ domains are 80-90 amino acids in length and typically recognize short peptide sequences at the C-terminus of their interacting partners. The PDZ domains of GRIP1 interact with the C-terminus of the cytoplasmic domain of FRAS1 and FREM2, mediating the cell-matrix interaction through a role in the trafficking of these cell surface ECM molecules (Fig. 1.9). GRIP1 is necessary for correct FRAS1 and FREM2 presentation, which in turn might be required for the recruitment of NG2 (CSPG) and collagens V and VI from the dermis to the basement membrane region.

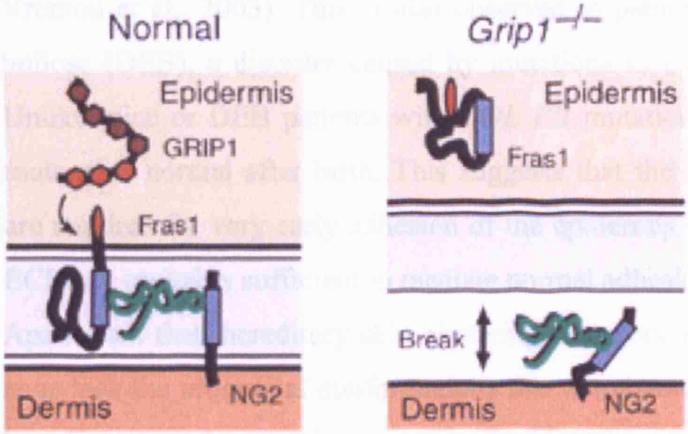


Figure 1.9: GRIP1 multiple PDZ domains and FRAS1

Model showing GRIP1-dependent transport of FRAS1 (and FREM2) to the basal epidermis and proposed interactions with collagen V and VI (green) and NG2 (Takamiya et al., 2004)

1.4.7 Different FREM proteins domains

FRAS1 has motifs at the N-terminus that are not found in ECM3 and in other FREM proteins. These motifs show a strong similarity to a number of proteins containing the cystein rich furin domains and Von Willebrand C domains, overlapped by chordin-/ kielin-like domains (Fig 1.6). Furin domains have been associated with modulation of

BMP and other TGFß related protein activity suggesting that FRAS1 might modulate their activity within the extra cellular matrix (McGregor et al., 2003).

FREM1 shares a number of structural domains with FRAS1 and FREM2 but misses the transmembrane or intracellular domains that allow direct interaction with GRIP1. FREM1 also lacks the furin and von Willebrand C domains that are present in FRAS1, but it has an additional C-terminal lectin type C domain.

1.4.8 Fras1/Frem/Grip1 pathway

Analysis of the *bl, my, Grip and heb/ bat* mutants, revealed that the fluid filled skin blebs characteristic of the blebbing mutants are first evident at embryonic day (E) 11.5 and that the plane of cleavage is below the basement membrane separating the dermis from the epidermis (McGregor et al., 2003; Smyth et al., 2004; Takamiya et al., 2004; Vrontou et al., 2003). This is also observed in patients with dystrophic epidermolysis bullosa (DEB), a disorder caused by mutations in *COL VII* (Christiano et al., 1993). Unlike mice or DEB patients with *COL VII* mutations, the epidermis of the blebbing mutants is normal after birth. This suggests that the FRAS/FREM and GRIP proteins are required for very early adhesion of the epidermis and that other components of the ECM are probably sufficient to mediate normal adhesion at later developmental stages.

Apart from that, hereditary skin blistering disorders affecting the basement membrane zone lack the urogenital malformations that are characteristic for the blebbing mutants. Renal agenesis becomes apparent in the blebbing mutants around E11.5 when highly apoptotic metanephric mesenchyme surrounding the ureteric bud is observed. Interruption of the extra cellular matrix proteins interactions seems to result in most of the observed anomalies in the blebbing mutant. Cryptophthalmos and syndactyly arise as a consequence of disturbed interactions of epidermal epithelia (eyelid and limb AER respectively) and the underlying mesenchyme. Impaired interactions at the interface between the ureteric bud and metanephric mesenchyme can result in renal agenesis. The multi domain structures of FRAS1 and FREM proteins suggest that they can interact with many different components of the ECM.

1.5 Aims of the project

The aim of this project was to describe the clinical findings in 59 FS patients and to evaluate the existing diagnostic criteria, to perform mutation analysis of the genes known to be involved in FS, to perform genotype-phenotype analysis and to complete linkage analysis of candidate genes for Fraser syndrome. Patients are described in Chapter 2, the results of these investigations are described in Chapter 3 and Chapter 4 and discussed in Chapter 5. The thesis concludes with a discussion of the findings and suggestions for future research.

Chapter 2

Materials and Methods

Chapter 2 Materials and Methods

2.1 Patients

2.1.1 Patient samples

Patients and families studied in this project were acquired in several ways. A number of families were already included in Lesley McGregor's PhD project and were seen by clinicians within the Clinical Genetics Unit at Great Ormond Street Hospital, or acquired through collaborations with other Clinical Genetics units both within the UK and worldwide. New patients were ascertained following publications on *FRAS1* and *FREM2* (Jadeja et al., 2005; McGregor et al., 2003).

Patients were diagnosed with Fraser syndrome by the referring clinician according to the current diagnostic criteria described in section 1.1.1. Where possible, clinical photographs, extensive clinical information and post-mortem reports were obtained. Referring clinicians are listed in table 2.1.

For most of the patients and their families, clinical data, and DNA samples were available for laboratory analysis. In the case of deceased children, paraffin tissue embedded samples were sent for DNA extraction.

For the work described in this thesis, clinical data, DNA samples or tissue samples were obtained from a total of 59 affected individuals from 25 consanguineous and 15 non-consanguineous families.

The project has been given ethical approval after review by the Great Ormond Street Hospital for Children NHS Trust/ Institute of Child Health Research Ethics Committee (reference number 1350).

Family	Referrer	Hospital	Contact
1	Bronwyn Kerr	United Kingdom	Bronwyn.kerr@cmmc.nhs.uk
2	Andre Megarbane	Beirut	megarbane@usj.edu.lb
3	Nicole Philip	France	nicole.philip@mail.ap-hm.fr
4	Angus Dobbie	United Kingdom	angus.dobbie@leedth.nhs.uk
5	Ton van Essen	The Netherlands	a.j.van.essen@medgen.azg.nl
6	John Nelson	Australia	John.Nelson@health.wa.gov.au
7	Jose Ferreira	Portugal	zec62@netvisao.pt
8	Perez Aytes	Spain	perez_ant@gva.es
9	Nicole Philip	France	nicole.philip@mail.ap-hm.fr
10	Peter Meinecke	Germany	meinecke@uke.uni-hamburg.de
11	Christine Francannet	France	c.francannet@chu-clermontferrand
12	Sue Holder	United Kingdom	s.holder@imperial.ac.uk
13	Willie Reardon	Ireland	wreardon@olhsc.ie
14	Dian Donnai	United Kingdom	Dian.Donnai@CMMC.nhs.uk
15	Dian Donnai	United Kingdom	Dian.Donnai@CMMC.nhs.uk
16	Arianna Bonato	Spain	aryred@hotmail.com
17	Brigitte Gilbert	France	b.gilbert@chu-poitiers.fr
18	Andew Wilkie	United Kingdom	awilkie@worf.molbiol.ox.ac.uk
19	David Chitayat	Toronto	dchitayat@rogers.com
20	David Chitayat	Toronto	dchitayat@rogers.com
21	Heiko Reutter	Germany	reutter@uni-bonn.de
22	Brigitte Gilbert	Poitiers	b.gilbert@chu-poitiers.fr
23	Lihadh Al-Gazali	UAE	l.algazali@uaeu.ac.ae
24	John Tolmie	United Kingdom	John.Tolmie@yorkhill.scot.nhs.uk
25	Morel	Canada	Serge.melancon@muhc.mcgill.ca
26	Frances Elmsie	United Kingdom	Frances.elmslie@stgeorges.nhs.uk
27	Eva Todos	Private	eva.yap.todos@gmail.com
28	JohnGraham	USA	John.Graham@cshs.org
29	Peter Meinecke	Germany	meinecke@uke.uni-hamburg.de
30	John Graham	USA	John.Graham@cshs.org
31	David Chitayat	Toronto	dchitayat@rogers.com
32	Christel Thauvin	France	christel.thauvin@chu-dijon.fr
33	Raoul Hennekam	London	I.B.Mathijssen@amc.uva.nl
34	Alina Midro	Poland	midro@amb.edu.pl
35	Heloise Santos	Portugal	
36	Raoul Hennekam	United Kingdom	r.hennekam@ich.ucl.ac.uk
37	Saskia Maas	The Netherlands	s.kapma@amc.uva.nl
38	Louise Wilson	United Kingdom	L.Wilson@ich.ucl.ac.uk
39	John Graham	USA	John.Graham@cshs.org
40	John Graham	USA	John.Graham@cshs.org

Table 2.1: List of referring clinicians

2.1.1.1 Clinical details of consanguineous families

Pedigrees are illustrated only if families were consanguineous. If clinical pictures were available, they will be added to the description of the affected family.

Family 1

This is a first cousin consanguineous pedigree with one healthy and one affected child who died shortly after birth from a laryngeal atresia that had already been detected on a prenatal scan at twenty weeks of gestation. The mother was karyotyped following repeated miscarriages and found to carry a balanced chromosomal translocation; 46,XX t(2;16)(p15;q22). The same balanced translocation was found in the affected child. The female infant had right-sided anophthalmos, malformed ears, a right sided nasal cleft with an intact palate, unilateral syndactyly of the hands, bilateral single palmar creases, fused labia and an imperforate anus.

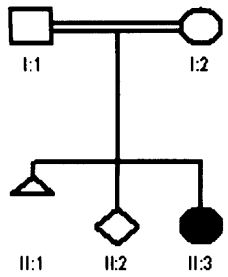
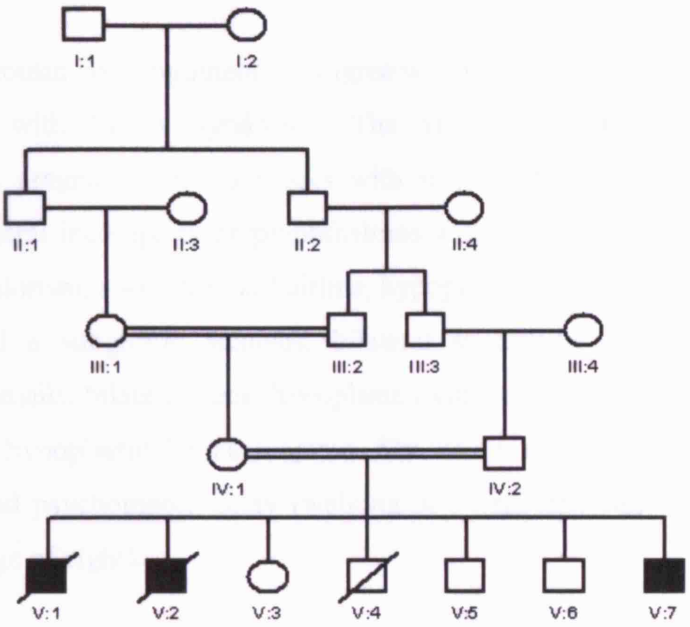


Figure 2.1: Pedigree of family 1

Family 2

This is a first cousin consanguineous pedigree with three affected sibs (of whom two died), three healthy sibs and one unaffected child who died at the age of 6 years of unknown cause. The affected male child (V:7) is alive and well. This child has bilateral crypthophthalmos (complete on the left and incomplete on the right), oral frenulae, widely spaced nipples, unilateral renal agenesis, unilateral inguinal hernia, and anal atresia. One of the other affected boys (V:2) who died postnatally had a meningocele and syndactyly, unfortunately there is no other information on the deceased affected children.

a]



b]

c]

Figure 2.2: Pedigree and clinical pictures of family 2

(a) Pedigree (b) affected boy (V:7) with bilateral syndactyly of hands and feet, unilateral inguinal hernia (c) complete cryptophthalmos of left- and partial cryptophthalmos of the right eye.

Family 3

This is a first cousin consanguineous pedigree with two female siblings, one healthy and one affected with Fraser syndrome. The affected female was born after an uncomplicated pregnancy of 36 weeks with microcephaly and facial dysmorphism including bilateral incomplete cryptophthalmos with unilateral coloboma of the upper eyelid, hypertelorism, a low frontal hairline, hypoplastic alae nasi and small, malformed ears. She had a subglottic stenosis, bilateral syndactyly of the hands and feet, hypoplastic toenails, bilateral renal hypoplasia (without functional anomalies at the age of eight) and hypoplastic labiae majorae. She has a significant hearing loss, visual impairment and psychomotor delay (walking at 30 months and speaking only a few words at the age of eight).

Figure 2.3: Pedigree and clinical pictures of family 3
(a) Pedigree (b) facial features of affected patient, (c) syndactyly of the hands

Family 4

This is a multiple consanguineous pedigree with two separate marriages that have each produced an affected child. Both patients are alive and well.

The male child (V:3) has incomplete cryptophthalmos on the right, complete on the left, bilateral cutaneous syndactyly of the hands and feet, unilateral renal agenesis, unilateral ureterocele, and hypospadias. He had a stridor at birth, (which has resolved with age and may have been related to a mild laryngeal stenosis), gastro-oesophageal reflux and bilateral sensori-neural deafness. He had a parietal skull defect with underlying

herniation of a skin-covered brain defect. CT scan of the brain showed a parietal schizencephaly.

The affected girl (V:12) in this family has a large occipital skull defect, bilateral cryptophthalmos with left sided anophthalmos, low set ears, a notched nasal tip with small nostrils, bilateral syndactyly of hands and feet, unilateral renal agenesis, and ambiguous genitalia with cliteromegaly. She is reported to have a hoarse cry.

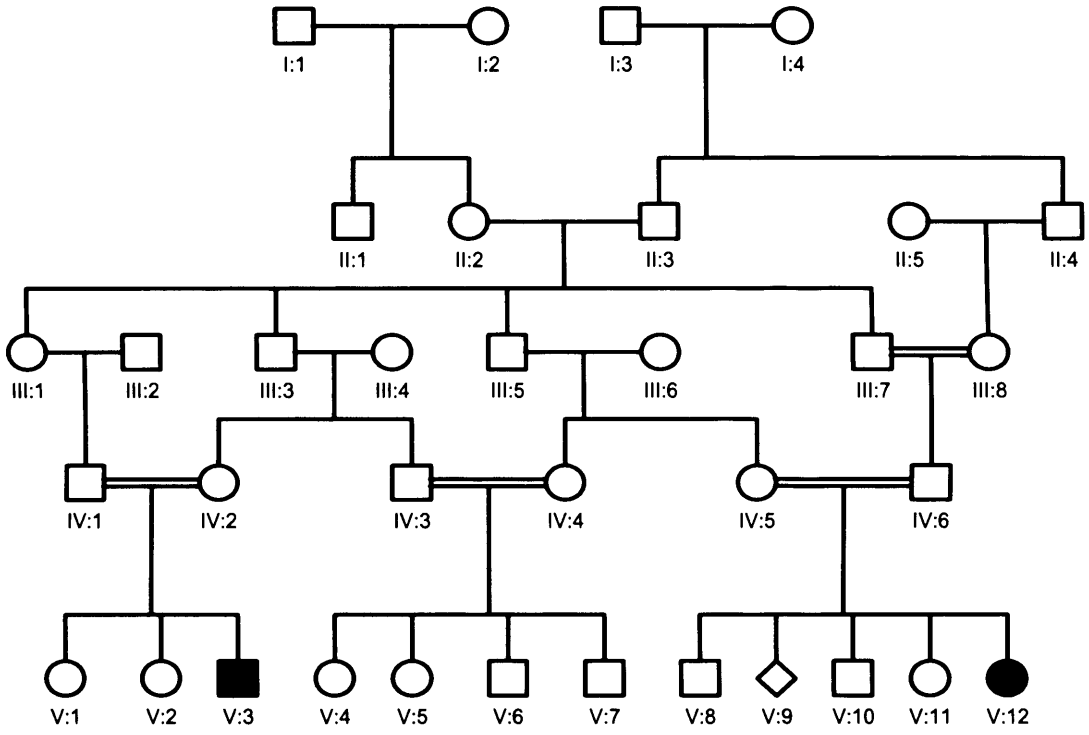


Figure 2.4: Pedigree of family 4

Family 5

This is a third cousin consanguineous family. The first child is a healthy girl. The second pregnancy was complicated by oligohydramnios and intrauterine growth retardation. An affected boy was born after 34 weeks of gestation. He died soon after birth. He had bilateral partial cryptophthalmos, with fusion of the right lower eye lid to the cornea and left anophthalmos, a unilateral cleft lip and palate, dysplastic low set ears, proximal and distal contractures of the limbs, partial cutaneous syndactyly of hands and feet with short halluces, a low inserted umbilicus and anal atresia. Post-mortem examination revealed a laryngeal stenosis and bilateral renal agenesis. A skeletal survey showed wide symphysis pubis. Chromosomes were normal (46,XY).

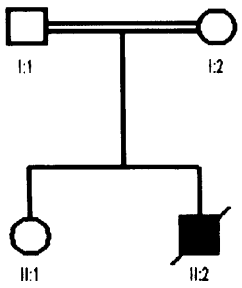


Figure 2.5: Pedigree of family 5

Family 6

This is a consanguineous pedigree. Parents are first cousins and had ten pregnancies, including two miscarriages. There are three healthy siblings and there have been five terminations for Fraser syndrome. Clinical information is available on four of these terminated foetuses.

The fourth pregnancy (VI:4) was terminated at 18 weeks of gestation due to multiple congenital abnormalities detected on prenatal scans. Postnatal examination showed a male foetus, with bilateral cryptophthalmos, unilateral microphthalmia, a grooved nasal bridge, low set dysplastic ears, laryngeal stenosis, bilateral cutaneous syndactyly of hands and feet, pterygia of elbows and knees, bilateral renal agenesis, absence of

ureters, bladder and urethra, ambiguous genitalia with a vestigial penile shaft. An X-ray of the thorax showed bilaterally only 11 ribs.

The fifth pregnancy (VI:5) was terminated at 30 weeks of gestation after prenatal ultrasound showed multiple congenital anomalies. Post-mortem examination showed complete left-sided cryptophthalmos with a hair line extending to the eye pit, incomplete right-sided cryptophthalmos, up slanted eyelids, low set ears with a possible incomplete external meatus on the left, right temporal bone defect, high arched palate, tracheo- oesophageal fistula, laryngeal stenosis, abnormal lung lobulation, preductal aortic stenosis, cutaneous syndactyly of hands and feet, mild hypoplasia of the finger nails and severely hypoplastic toe nails, bilateral single palmar creases, bilateral renal agenesis, absence of the ureters, ambiguous genitalia, absence of the vagina and uterus but streak ovaries present inside the pelvis.

The sixth pregnancy (VI:6) ended in a termination at 19 weeks of gestation because of prenatally detected congenital abnormalities that were consistent with Fraser Syndrome. Post-mortem examination revealed a male foetus with bilateral cryptophthalmos, posteriorly rotated low set ears, laryngeal stenosis, bilateral enlarged lungs with deficient fissures, cutaneous syndactyly of hands and feet, low inserted umbilical cord with only 2 vessels, bilateral renal agenesis, anal atresia, a small penis, hypoplasia of the scrotum and inverted talipes of the feet.

The seventh pregnancy (VI:7) was terminated at a gestation of 16 weeks. The male child had an abnormally shaped skull (oxycephaly), bilateral cryptophthalmos, broad nose trills, low set dysplastic ears, nuchal oedema and oedema extending to below the chin, laryngeal stenosis, cutaneous syndactyly of hands and feet, duodenal atresia, agenesis of the kidneys and bladder, male genitalia with hypoplastic scrotum, and an imperforate anus. A chest X-ray showed bilateral only 11 ribs.

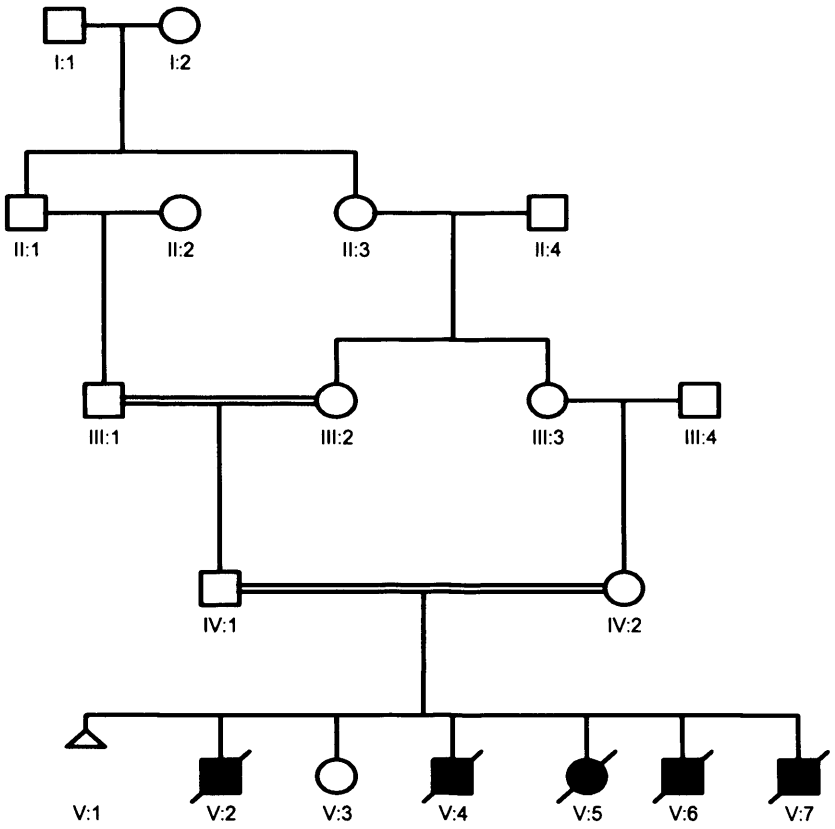


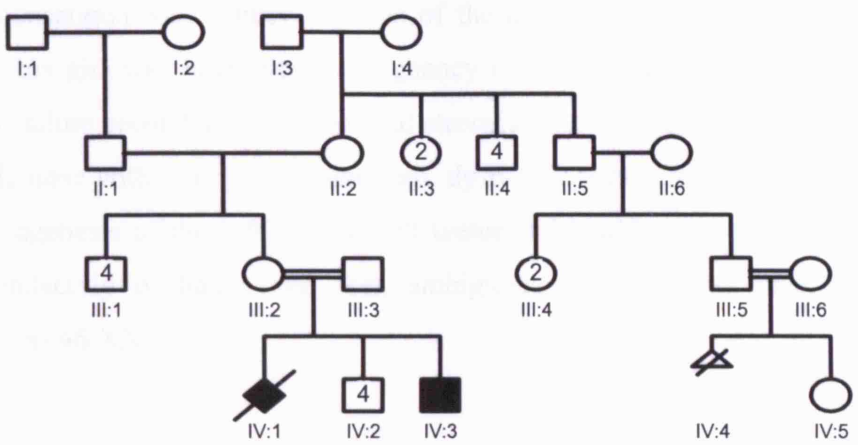
Figure 2.6: Pedigree of family 6

Family 7

This is a multiply inbred pedigree with several affected Fraser patients. The first couple has 3 healthy and 2 affected children. No information is available on the first affected child. The second affected boy (IV:3) was born after a term pregnancy with a blepharophimosis and a laryngomalacia for which he has been treated. He has cutaneous syndactyly and typical facial features of Fraser Syndrome (Fig. 2.7).

Another consanguineous couple in this family has two healthy children and had a termination of a third pregnancy (IV:4) after a prenatal ultrasound at 14 weeks had detected an encephalocele and syndactyly. Post-mortem examination confirmed the prenatal findings and revealed a unilateral blepharophimosis.

a]



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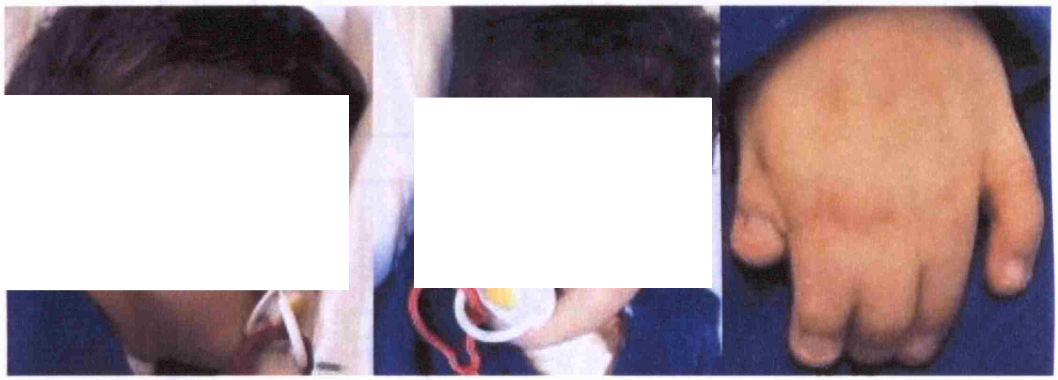


c]

Figure 2.7: Pedigree and clinical pictures of family 7

(a) Pedigree (b) affected boy (IV:3) (c) affected foetus (IV;4)

Family 8

This is a consanguineous family. Parents of the affected child are first cousins once removed. This girl was born after a pregnancy of 32 weeks and died at birth due to respiratory failure secondary to a laryngeal stenosis. She had bilateral cryptophthalmos, a dysplastic nose with hypoplastic alae nasi, dysplastic ears, a short thorax, abdominal distension, agenesis of the left kidney, left ureter and bladder, hypoplasia of the right kidney, syndactyly of hands and feet, ambiguous genitalia and anal atresia. The karyotype was 46,XX.

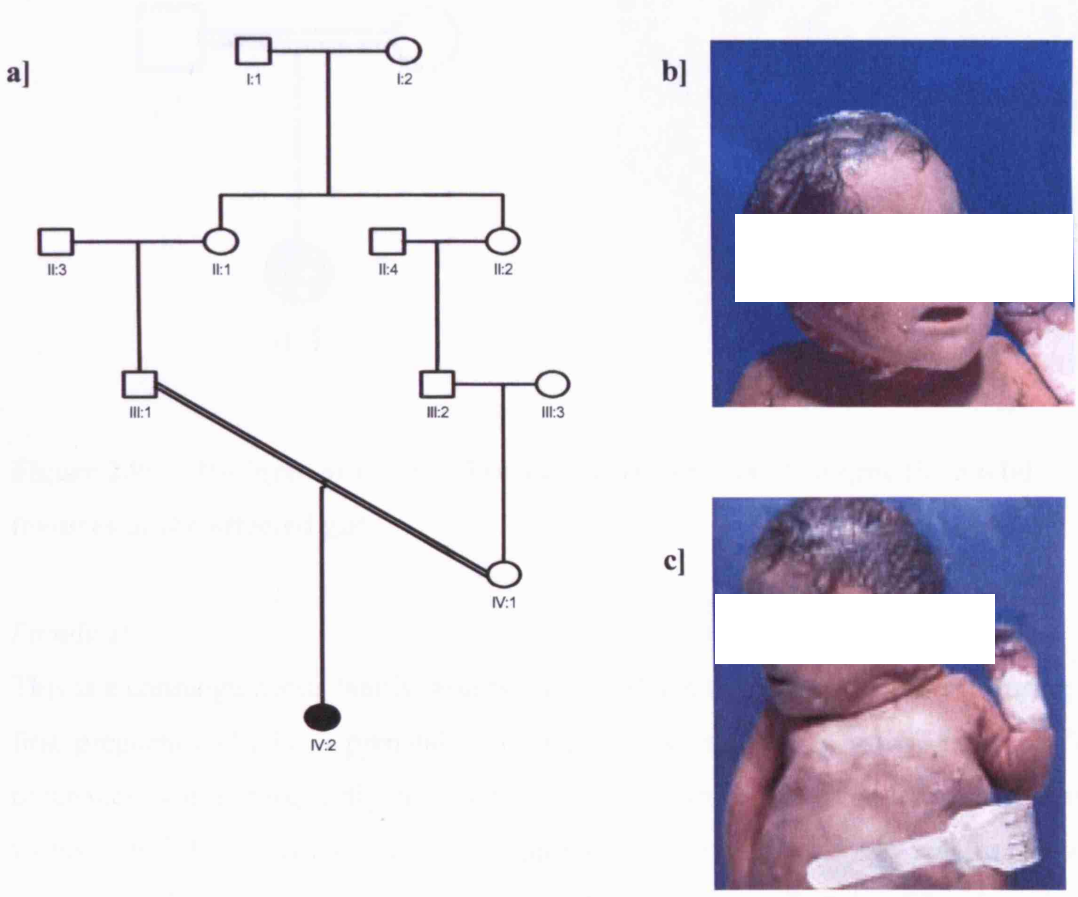


Figure 2.8: Pedigree and clinical pictures of family 8

(a) Pedigree (b) clinical features of the affected foetus (IV:2), beak shaped nose, bilateral cryptophthalmos (c) syndactyly of the left hand, low set dysplastic ear

Family 9

This is a consanguineous family. Parents of the affected girl are second cousins. She was born after an uncomplicated pregnancy of 35 weeks. She has a right-sided cryptophthalmos and a malformed left globe, abnormal anterior hairline, small mouth, bilateral partial syndactyly of the hands, unilateral partial syndactyly of the feet and ambiguous genitalia.

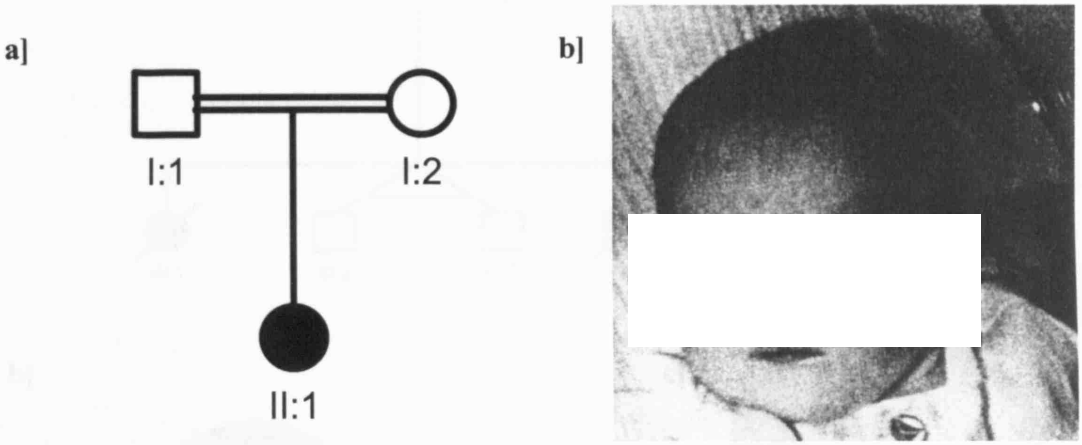


Figure 2.9: Pedigree and clinical picture of family 9 (a) Pedigree (b) Facial features of the affected girl

Family 10

This is a consanguineous family with two affected and two healthy children. During the first pregnancy (III:1), a prenatal scan at 22 weeks showed a Potter sequence. The pregnancy was subsequently terminated. Post-mortem examination showed a female foetus with bilateral coloboma of the upper eyelids, hypertelorism, a small nose with broad nasal bridge, small dysplastic ears, and cutaneous syndactyly of hands and feet. Autopsy revealed a laryngeal stenosis, hypoplastic lungs, and agenesis of the kidneys and uterus.

The second pregnancy led to healthy monozygous twin brothers. An affected male (III:4) was born after the third pregnancy. He died soon after birth due to severe laryngeal stenosis, which led to severe respiratory difficulties. Physical examination

showed a male child with unilateral cryptophthalmos, a flat and wide nasal bridge, hypoplastic alae nasi, low set, small and simple shaped ears, a low set umbilicus, a small omphalocele and syndactyly of the feet with hypoplastic toenails (Fig. 2.10). Abdominal sonography revealed absent kidneys, a chest X-ray showed hypoplastic-collapsed lungs. He had a normal male karyotype (46, XY).

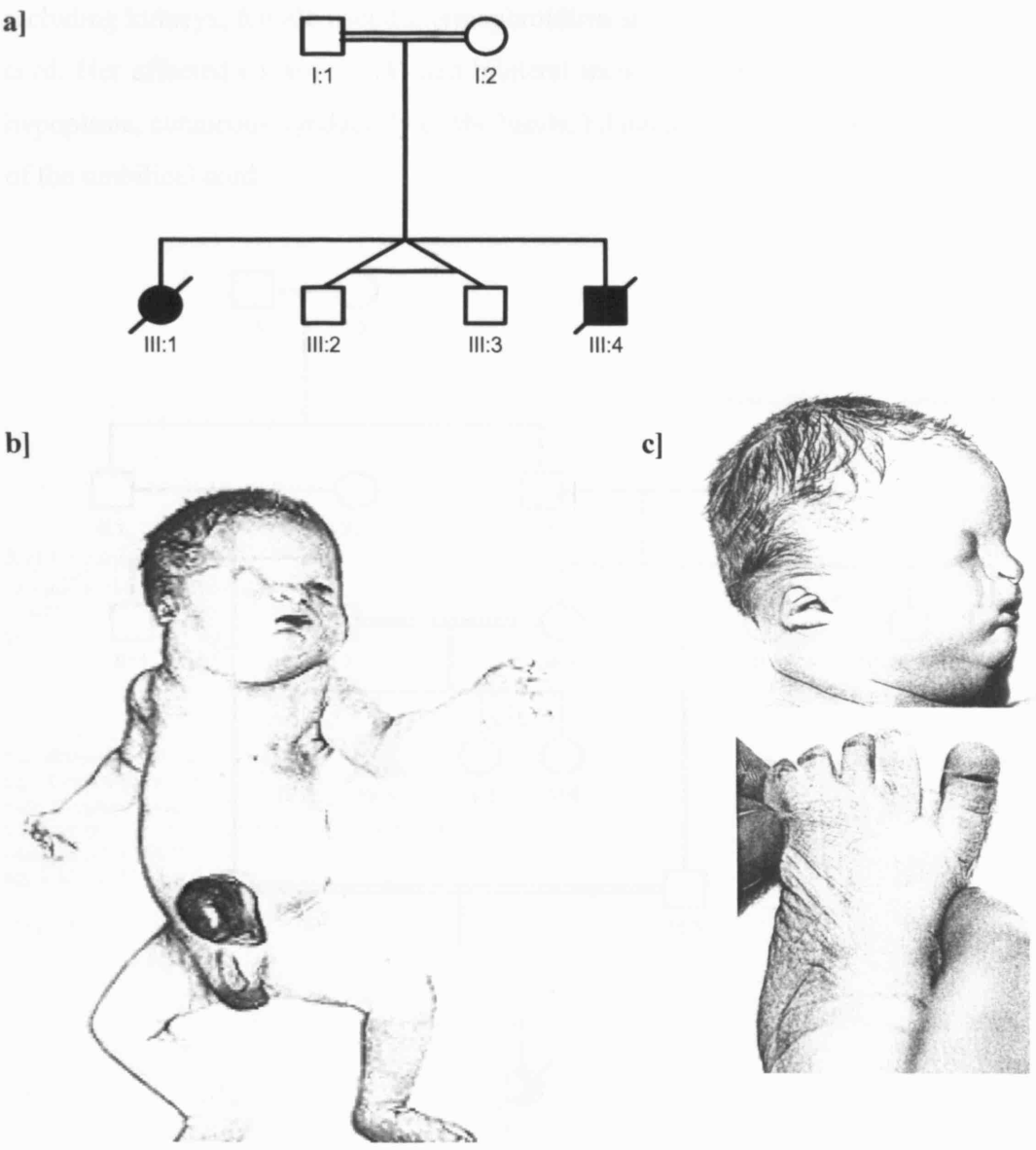


Figure 2.10: Pedigree and clinical pictures of family 10

(a) Pedigree, (b) affected foetus (III:4) with omphalocele (c) low set ears and hypoplasia of the nails

Family 11

This is a large consanguineous Turkish family with two affected cousins, who both died postnatally. The affected girl (IV:2) died at 36 weeks of gestation due to severe oligohydramnios. She had no cryptophthalmos but synechiae between the cornea and eyelids, cutaneous syndactyly of the hands, complete agenesis of the urinary tract including kidneys, female pseudohermaphroidism and low implantation of the umbilical cord. Her affected cousin (IV:4) had bilateral incomplete cryptophthalmos, pulmonary hypoplasia, cutaneous syndactyly of the hands, bilateral renal agenesis and low insertion of the umbilical cord.

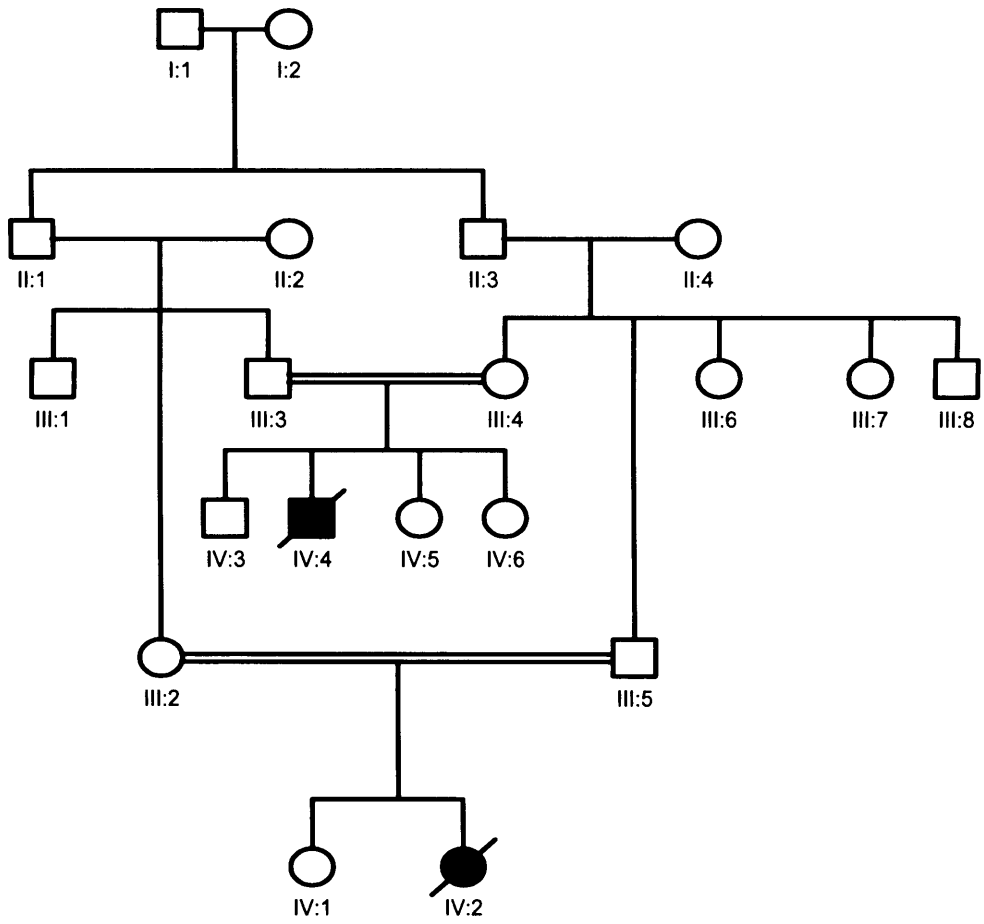


Figure 2.11: Pedigree of family 11

Family 12

This affected female is the third child of consanguineous parents (first cousins). She has bilateral complete cryptophthalmos, unilateral choanal atresia, low set ears, cutaneous syndactyly of the hands, unilateral 2/3 syndactyly of the toes, unilateral renal agenesis, low insertion of the umbilical cord, ambiguous genitalia, and an imperforate anus with a recto-vaginal fistula. She is reported to have a severe conductive hearing loss.

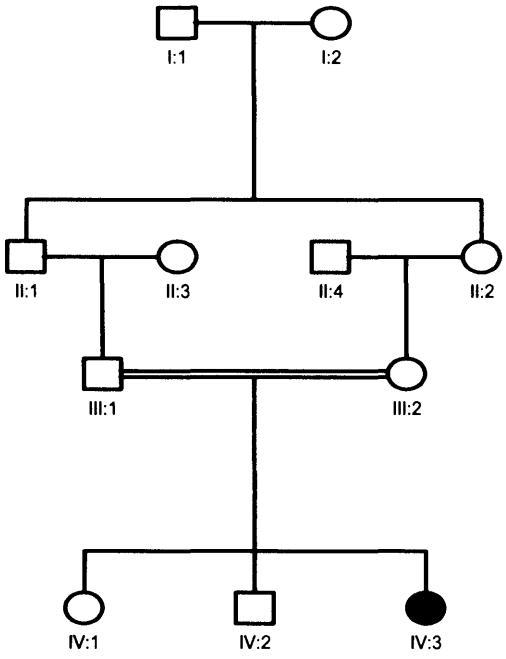


Figure 2.12: Pedigree of family 12

Family 13:

This is a multiply inbred family. The affected female is the first child of consanguineous (first cousins) parents. She has bilateral cryptophthalmos, malformed rudimentary ears, micrognathia, syndactyly of the hands, ambiguous genitalia (hypoplastic labia and cliteromegaly), and an anteriorly placed anus.

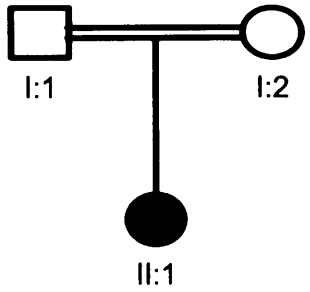


Figure 2.13: Pedigree of family 13

Family 14:

This affected female is the product of a father daughter mating. She has microphthalmia, a hypoplastic nose, simple ears, severe laryngeal stenosis, digital abnormalities of all four limbs, a single umbilical artery, unilateral renal agenesis, genital abnormalities, a skin abnormality that is not further described, and anal atresia.

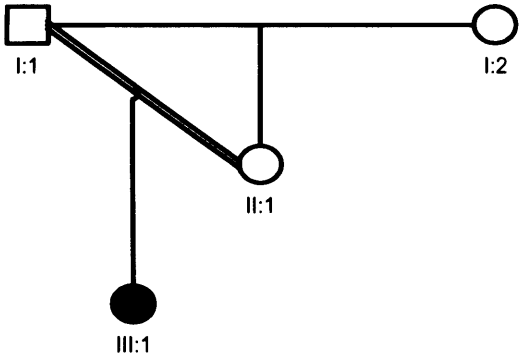


Figure 2.14: Pedigree of family 14

Family 15

This is a multiple consanguineous family of Irish gypsies. The sibship consists of six healthy and three affected children of whom two died postnatally and one was still born). The first affected female (II:6) had bilateral cryptophthalmos, low set ears, laryngeal atresia, atrial septal defect, cutaneous syndactyly of hands and feet, low

inserted umbilical cord, unilateral renal agenesis, imperforate anus, ambiguous genitalia and an imperforate vagina with hydrocolpos. The second affected child (II:7) has complete cryptophthalmos on the right and incomplete cryptophthalmos on the left side, small malformed ears, microstomia, bilateral syndactyly of hands and feet, ambiguous genitalia and an anteriorly placed anus. The stillborn affected child (II:8) was part of a twin; her healthy twin brother is still alive. She had bilateral complete cryptophthalmos, cleft lip, rudimentary nose, small posteriorly rotated ears, 2/3 syndactyly of the left hand, complete syndactyly of the right hand with a small thumb, and ambiguous genitalia.

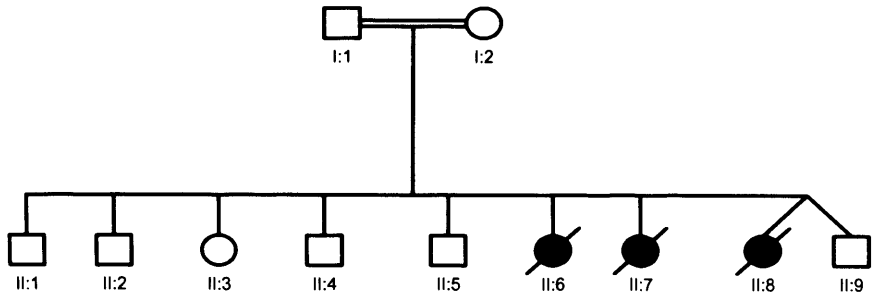


Figure 2.15: Pedigree of family 15

Family 16

The first pregnancy (V:1) of this consanguineous couple (Spanish Gypsies) ended in a termination, after an antenatal scan at 18 weeks had shown congenital abnormalities (severe oligohydramnios, bilateral pulmonary hypoplasia, renal agenesis and absence of the bladder). Post-mortem examination was difficult due to severe maceration of the foetus. Apart from the above mentioned anomalies. There was also syndactyly of the hands.

The second pregnancy ended with a miscarriage at 12 weeks. Pathological findings did not reveal congenital malformations.

Ultrasound findings of the third pregnancy (V:3) at 19 weeks showed anhydramnios, tracheal obstruction, lung abnormalities (cystic adenomatoid malformation type III), hepatomegaly, ascites, placentomegaly and agenesis of the kidneys and bladder. Chromosomal analysis revealed a normal female karyotype (46,XX).

Post-mortem examination showed right-sided cryptophthalmos and micropthalmia, a normal left eye, a hypoplastic broad nose, low set and small dysplastic ears with atresia of the left ear canal, a short and oedematous neck, syndactyly of the hands and feet, ambiguous genitalia (cliteromegaly) and an imperforate anus. Internal examination showed hypoplasia of the epiglottis, thymus and larynx, a dysplastic upper part of the trachea (with macroscopic absence of the thyroid cartilage and thymic gland), hyperplastic lungs with abnormal lobulation (only 1 left and 3 right lobules), intestinal malrotation, bilateral renal agenesis, a hypoplastic bladder, rectal atresia with a possible fistula to the cervix; dysgenesis of ovaries and a uterine malformation (cloaca?).

A maternal uncle had a son (IV:3) with anhydramnios, dysplastic/ hypoplastic cystic kidneys, rectal atresia and imperforate anus.

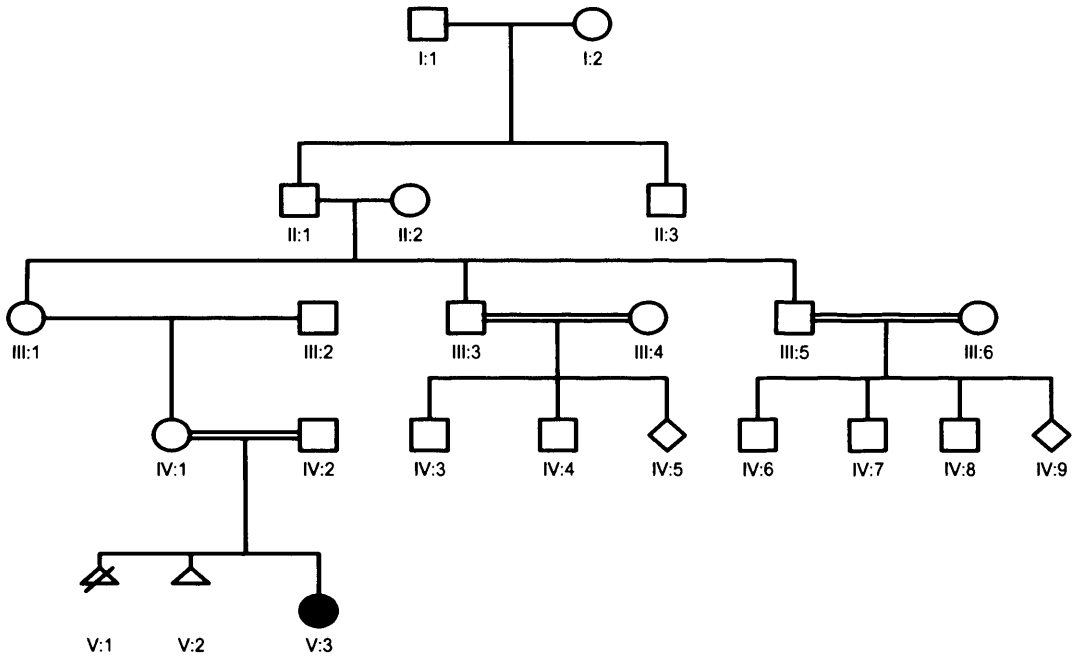


Figure 2.16: Pedigree of family 16

Family 17

The affected male foetus (III:7) is the first pregnancy of consanguineous parents. Prenatal scanning showed anhydramnios, bilateral renal agenesis and abnormal position of the feet. The pregnancy was terminated at 23 weeks. Post-mortem examination

showed facial dysmorphism; partial right-sided cryptophthalmos, left-sided microphthalmia, hypertelorism, flat nose, unilateral cleft lip and hypoplastic ears. He had limited abduction of the limbs, pterigium of the axillaries, syndactyly of hands and feet, clubfeet, normal male genitalia, and anal atresia.

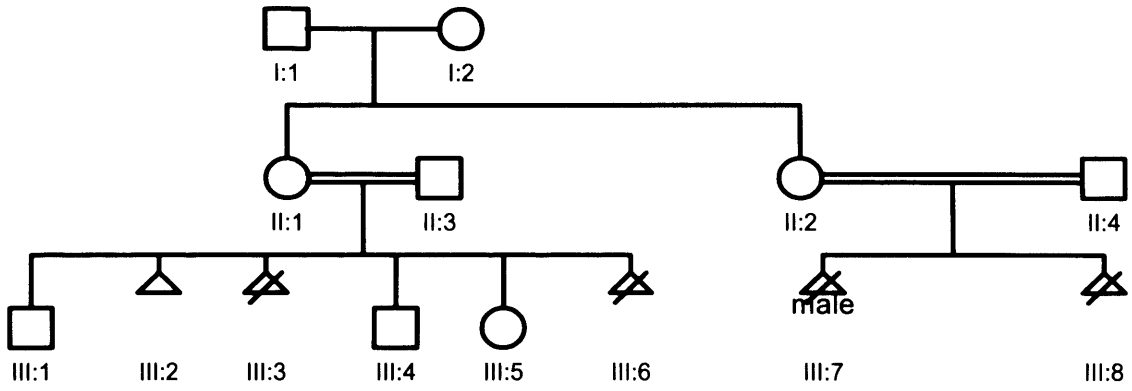


Figure 2.17: Pedigree of family 17

Family 18

This is a third cousin pedigree with one affected child. The child has complete cryptophthalmos on the left- and incomplete cryptophthalmos on the right side, low-set malformed ears, bilateral cutaneous syndactyly of hands and feet, and ambiguous genitalia.

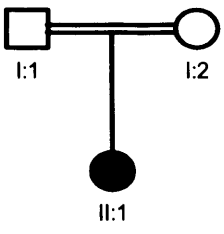


Figure 2.18: Pedigree of family 18

Family 19

The first pregnancy of this consanguineous couple ended in a termination in the second trimester after a prenatal ultrasound had detected multiple congenital malformations suggestive of Fraser syndrome. Post-mortem examination showed a male foetus with a small nose, low set ears, bifid uvula, tracheal stenosis, pulmonary hyperplasia, bilateral syndactyly of hands and feet, low insertion of the umbilical cord, agenesis of the left-, and hypoplasia of the right kidney, and ambiguous genitalia with bilateral intra-abdominal testes. Cryptophthalmos was not present. A skeletal survey showed microcephaly, syndactyly and low set ears.

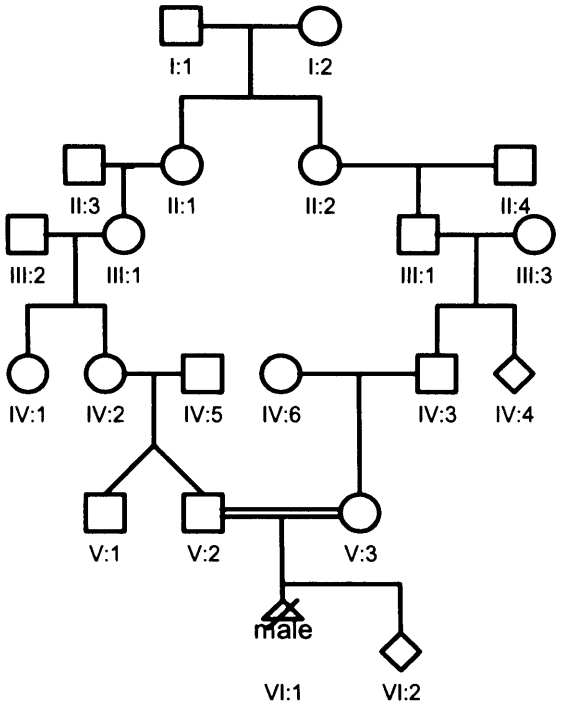


Figure 2.19: Pedigree of family 19

Family 20

This is a consanguineous family (parents are first cousins), with one affected child and two healthy children. The affected male child was born after 39 weeks of gestation and died soon after birth. Post mortem examination showed that he was small for his age, and had an oligohydramnios sequence (redundant nuchal skin, large flattened ears, and

large spade-like hands). He had left sided cryptophthalmos, an ovoid limbus of the right eye, low set hypoplastic ears, syndactyly of the hands and feet, right renal agenesis, left renal hypoplasia, and abnormal external genitalia (hypospadias, bulbous prepuce and hypoplastic scrotum).

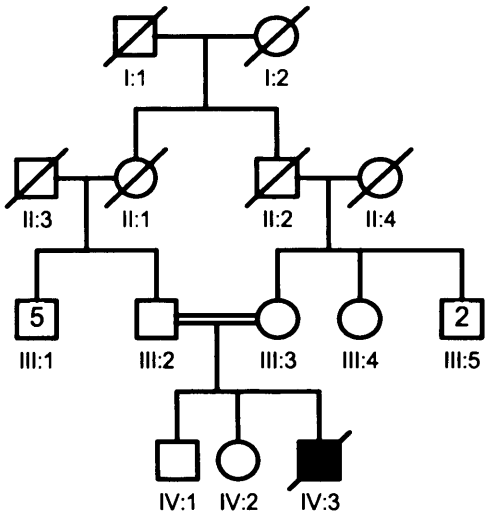


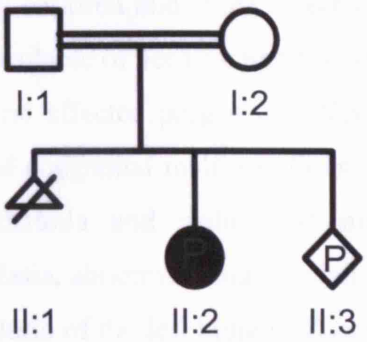
Figure 2.20: Pedigree of family 20

Family 21

This is a consanguineous pedigree. Parents are first cousins from a German travelling family. The first pregnancy was terminated after prenatal tests had shown that the foetus had an unbalanced translocation of chromosomes 18 and 21. The mother carried a balanced translocation of these chromosomes.

Antenatal scans during the second pregnancy showed congenital malformations fulfilling the diagnostic criteria for Fraser syndrome. The foetus had an intrauterine correction for a laryngeal atresia at 22 weeks of gestation. Postnatally, the child showed incomplete cryptophthalmos and microphthalmia on the left- and complete cryptophthalmos on the right side, an abnormally shaped nose, facial asymmetry, bilateral cutaneous syndactyly of hands and feet, and an omphalocele. Chromosome analysis revealed a normal female karyotype (46,XX).

a]



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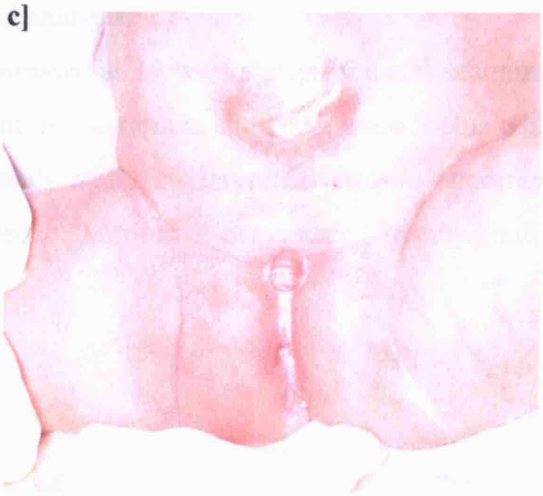

Figure 2.21: Pedigree and clinical pictures of family 21

(a) Pedigree (b) facial appearance of II:2 (c) low inserted umbilicus and ambiguous genitalia

Family 22

This is the first pregnancy of a consanguineous couple (second cousins from a Spanish Gypsy- family). The pregnancy was terminated at 22 weeks after prenatal scans showed congenital abnormalities. Post-mortem examination showed a foetus (V:3) with an oedematous face, incomplete cryptophthalmos and microphthalmia on the right, and partial fusion of the eyelids on the left side, a large bifid nose, a short philtrum, micrognathia, low set ears, tracheal stenosis, pulmonary hyperplasia, omphalocele,

bilateral syndactyly of the hands, bilateral clubfeet, renal agenesis, and ambiguous genitalia with bilateral intra-abdominal testes. The mother has a sister who has three healthy children and three affected pregnancies with Fraser syndrome. Information is only available of her first and last affected pregnancy.

The first affected pregnancy (V:4) was terminated at 30 weeks after prenatal scans showed congenital malformations. Post-mortem examination showed a foetus with left anophthalmia and right-sided microphthalmia, a cleft lip and palate, laryngeal hypoplasia, abnormal lung lobulation, agenesis of the thymus, agenesis of the right- and hypoplasia of the left kidney. There was syndactyly of the hands and feet. The external genitalia were ambiguous, however the gonads were in favour of a female. This has however not been confirmed by chromosomal analysis.

The third affected pregnancy (V:5) was terminated at 15 weeks after prenatal scanning had revealed a recurrence of FS. Post mortem investigations showed a male foetus with cryptophthalmos, bilateral syndactyly of hands and feet, laryngeal atresia, abnormal lung lobulation, an abdominal skin defect, malrotated mesentery, hypogenitalia (micropenis) and anal atresia.

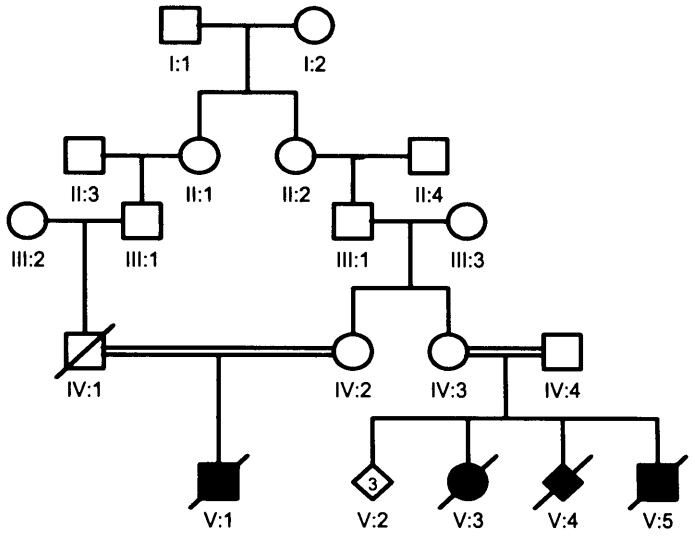

Figure 2.22: Pedigree of family 22

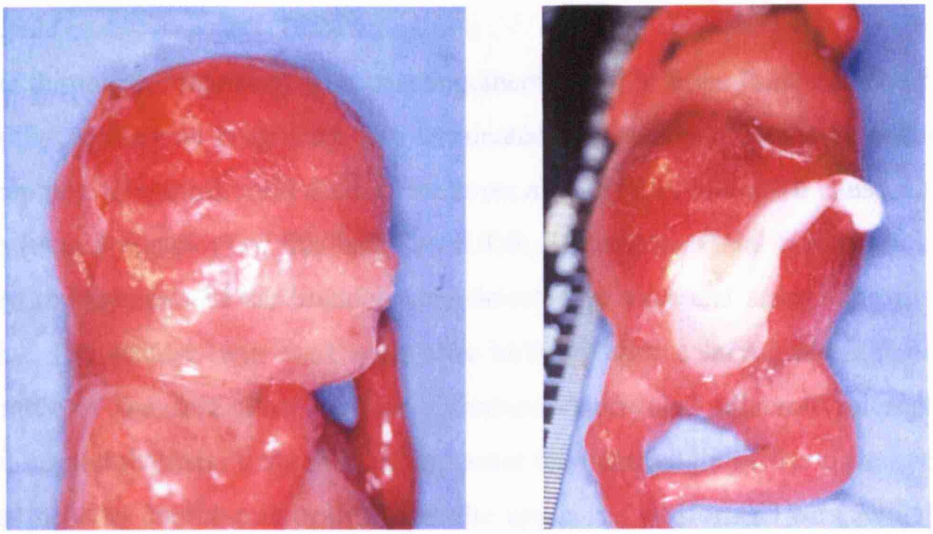

Figure 2.23: Clinical pictures of family 22; Affected foetus (V;5), note cryptophthalmos and abdominal skin defect.

Family 23

This is a first cousin consanguineous family with one healthy and four affected children who all died postnatally. Unfortunately clinical details are only available regarding the first and fourth affected child. The first affected child had bilateral cryptophthalmos, unilateral microtia, bilateral renal agenesis, syndactyly of the hands, and genital abnormalities (small phallus with cryptorchidism). The fourth affected child had right-sided cryptophthalmos and left sided microphthalmos, unilateral microtia with absence of the external ear canal, low insertion of the umbilical cord, fused labia, cliteromegaly, and arthrogryposis. Syndactyly was not present.

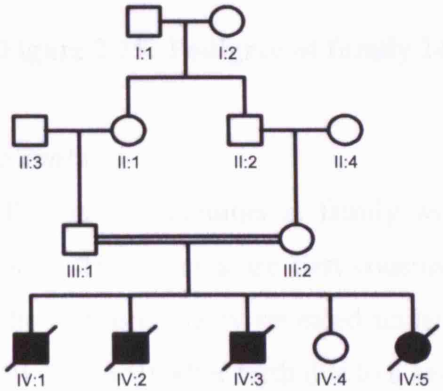

Figure 2.24: Pedigree of family 23

Family 24

This is the fourth pregnancy of a consanguineous couple from Pakistan. The first child is healthy; the second pregnancy was terminated at 26 weeks of gestation because of an anencephaly. The third child died a few hours after birth of unknown cause. During the fourth (twin pregnancy) of this baby, an IUGR, dysmorphic facial features, a horseshoe kidney and agenesis of the bladder were detected on antenatal scans. The first twin is healthy. The second twin died soon after birth. He had a laryngeal stenosis, flexion deformity of the feet. Post-mortem examination revealed bilateral microphthalmia, partial cryptohthalmos, a small skin tag under the outer angle of the right eye, a small beaked nose in continuous profile with the upper lip, malformed ears, widely spaced nipples, bilateral renal agenesis, discoid adrenal glands, small and hypoplastic bladder, unusually large penis, hypoplastic scrotum, flexion contractures of elbows and webbing of hands and feet.

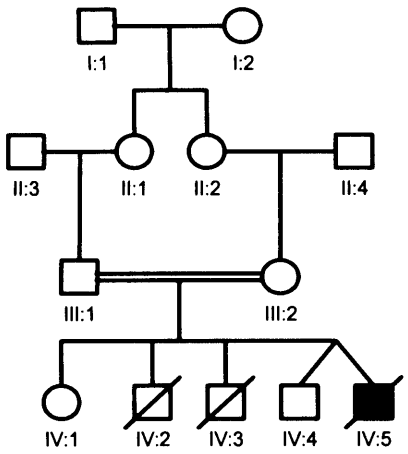

Figure 2.25: Pedigree of family 24

Family 25

This is consanguineous family with two affected children who both died soon after birth. The parents are first cousins of Pakistani background. Prenatal scanning during the first pregnancy revealed unilateral agenesis of the kidneys. The affected girl died (II:1) shortly after birth due to a severe laryngeal stenosis. Physical examination showed bilateral cryptophthalmos with proptosis and hypertelorism, a high arched palate,

bilateral external ear abnormalities, hypoplastic alae nasi, with notching and midline nasal cleavage, a low frontal hairline, with temporal extension of the hair onto the cheeks, partial cutaneous syndactyly of hands and feet and ambiguous genitalia. Post-mortem examination revealed a severe laryngeal stenosis, abnormal lung lobulation (1 lobe on the left, 2 on the right), cardiomegaly with a persistent ductus arteriosis and patent foramen ovale, widely spaced cranial bones with normal brain structures, a non-fixation of the right colon with coecum and appendix in the right upper quadrant, a 1 cm narrowing of the recto-sigmoid and a vaginal atresia.

Prenatal scanning during the second pregnancy (II:2) showed congenital abnormalities that suggested a recurrence of FS. The affected boy was born after a pregnancy of 36 weeks. He had bilateral cryptrophthalmos, a low hairline, depressed nasal bridge, small ears and cutaneous syndactyly. Abdominal examination showed agenesis of the right kidney and hypoplasia of the left kidney, a pneumothorax and pulmonary haemorrhage. The boy died two days after birth. The sister of the father also had a child who died of Fraser syndrome.

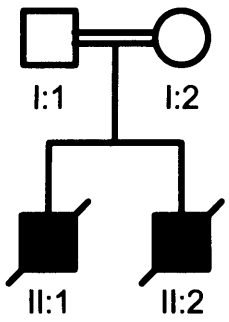

Figure 2.26: Pedigree of family 25

2.1.1.2 Clinical details of non-consanguineous families

Family 26

This is the third pregnancy of a non-consanguineous couple (who are from the same village in Pakistan). Renal agenesis was detected prenatally. The pregnancy was terminated at 24 weeks. Post-mortem examination showed a male foetus with bilateral

cryptophthalmos and microphthalmia on the left, a small, broad nose with a depressed nasal bridge, an extending hairline down onto the eyebrows, simple, low set ears, laryngeal stenosis, widely spaced nipples, bilateral partial cutaneous syndactyly of the hands, slightly webbed toes, renal agenesis, male genitalia with well formed penis but no development of a scrotal sac, and bilateral intra-abdominal testes. There were severe abnormalities in the spinal cord, cerebellum and aquaduct, all consistent with an underlying neural tube defect and Arnold Chiari malformation. The mother had two previous early miscarriages.

Family 27

This affected female is the first child of non-consanguineous parents. She has bilateral cryptophthalmos, small, low set ears with hypoplastic external ear canals, syndactyly of hands and feet, umbilical hernia, unilateral renal agenesis, ambiguous genitalia, vaginal atresia, and an atrial septum defect.

Figure 2.27: Pictures of family 27, note wide nasal bridge and cryptophthalmos.

Family 28

This is the first affected, third child of non-consanguineous parents. She has cryptophthalmos, bilateral aphacia, bilateral microtia with stenotic external ear canals, partial cutaneous syndactyly, tracheal and laryngeal, stenosis, extremely short true vocal cords, omega shaped, anteriorly placed epiglottis, narrow subglottic airway, flat nasal bridge with midline ridge on the nose, tight lingual frenulum, high arched palate, a common urogenital sinus, unilateral renal agenesis and a low inserted umbilical cord. She failed three times for an auditory brain stem response test.

Family 29

This is a 25 year-old son of non-consanguineous parents. He has a unilateral incomplete cryptophthalmos, syndactyly of hands and feet and a unilateral hypoplastic kidney. He has a normal IQ and attends university.

Figure 2.28: Pictures of family 29, note wide nasal bridge, unilateral cryptophthalmos covered by tongue of hair, and low set, simple shaped ears.

Family 30

This is the second child of non-consanguineous parents. He has a right-sided cryptophthalmos without a palpable eye, small left eye with medial coloboma, small dysplastic ears, a large left occipital skull defect, complete syndactyly of the hands and feet, bilateral single palmar crease, small laryngeal web, severe subglottic stenosis, mid-posterior tracheal cleft, tracheal and choanal stenosis and a unilateral renal agenesis.

Family 31

This is a severely affected foetus of non-consanguineous parents. Pregnancy was terminated in the second trimester. Post mortem examination revealed bilateral anophthalmia, low set ears, laryngeal stenosis, bilateral syndactyly of hands and feet, agenesis of the right kidney, multicystic dysplastic left kidney, a rudimentary phallus, and imperforate anus

Family 32

This is a non-consanguineous family with an affected child (32a) who died at birth after a pregnancy of 33 weeks. The child had complete bilateral cryptophthalmos, microphthalmia, a prominent forehead with widely spaced cranial bones, small malformed ears with canal stenosis, laryngeal stenosis, hypoplasia of the lungs, proximal shortening of the limbs, cutaneous syndactyly of the feet, bilateral renal agenesis, cliteromegaly and vaginal atresia.

The second pregnancy (32b) was terminated at 18 weeks because of recurrence of malformations detected at prenatal ultrasound scans. Amniocentesis showed a normal female chromosome pattern. Post-mortem investigations showed a prominent forehead, bilateral hypoplastic nostrils, low-set ears and bilateral asymmetric absent eyelids. There was syndactyly of the hands and feet. Abdominal examination showed bilateral agenesis of the kidneys and ureters, a rudimentary bladder and a low- set umbilicus.

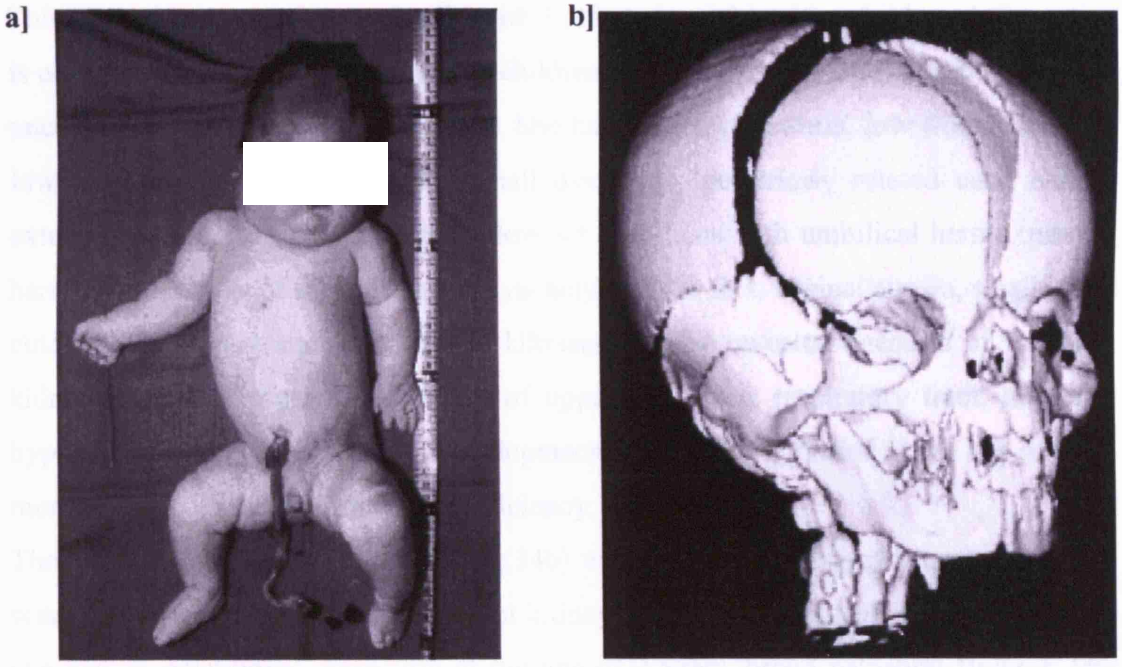

Figure 2.29: Clinical pictures of family 32,
(a) Patient 1, note complete bilateral cryptophthalmos, syndactyly of hands and feet, proximal shortening of the limbs, low set umbilicus, and cliteromegaly (b): 3D tomodensitometry bone reconstruction: widely spaced sutures. (Rousseau et al., 2002)

Family 33

This is a non- consanguineous family from the Netherlands with two affected sons. The first child (33a) died shortly after birth. He had bilateral cryptophthalmos, anophthalmia, malformed ears, bilateral cutaneous syndactyly of hands and feet, omphalocele, single umbilical artery, large bladder, intra abdominal testes, and anal stenosis. The karyotype was normal male (46,XY). Post mortem examination revealed an open ductus Bottali, open foramen ovale and hypoplasia of the lungs.

During the second pregnancy (33b), prenatal scans showed multiple congenital anomalies. The pregnancy was subsequently terminated at 14 weeks. Post mortem examination showed male foetus with a small mandible, broad hands, unilateral renal agenesis, umbilical hernia and a large bladder.

Family 34

This is a non-consanguineous family with 3 affected and 2 healthy children. Information is only available of two of the affected children. The first female (34a) was born after an uncomplicated pregnancy of 37 weeks. She had a microphthalmia, low frontal hairline, low nasal bridge, choanal atresia, small dysplastic, posteriorly rotated ears, narrow external ear canals, laryngeal atresia, low set umbilicus with umbilical hernia, narrow hands, clinodactyly of the Vth finger, syndactyly of the feet, vaginal atresia, small labia, clitoris hypertrophy and anal atresia. Ultrasonography revealed agenesis of the right kidney. She had recurrent infections of upper and lower respiratory tract, muscular hypotonia, failure to thrive and a developmental delay. The girl died at the age of three months due to cardio respiratory insufficiency.

The affected female who is still alive (34b) was born after a term pregnancy. Prenatal scans had revealed agenesis of the right kidney. She had a laryngeal stenosis for which she had an operational correction at the age of 2 years, broad palpebral fissures, low frontal hairline, broad nasal bridge, narrow mouth, small, posteriorly rotated low set ears, clinodactyly of the Vth fingers, partial cutaneous syndactyly of the feet, short halluces, a low set umbilicus with umbilical hernia, small labiae, clitoris hypertrophy and a cardiac defect (aneurysm, a IVS on foramen ovale level, a cord spurious and trabeculae carneae in LV, abnormal triscupitalis regurgitation, hypertrophy of septi

intraventriculare, extrasystole of ventriculi, hypercontractio LV, radiological examination showed accentation of truncus pulmonalis and LV).

Family 35

This is a mildly affected son of non-consanguineous parents. He has incomplete unilateral cryptophthalmos, a beaked nose, a low inserted umbilical cord and syndactyly of hands and feet. (Parents come from a small island so a founder effect might be present).

Family 36

This is the only child of non-consanguineous parents. She has bilateral cryptophthalmos for which she had an unsuccessful operation, a broad nose and unilateral renal agenesis.

Family 37

This is a non-consanguineous family. The affected boy was born after a pregnancy of 30 weeks. Prenatal scans had detected an omphalocele. The boy died shortly after birth. Post mortem examination revealed bilateral cryptophthalmos, absent olfactory nerves, severe cleft lip and palate, low set ears with atresia of the external ear canal, bilateral partial syndactyly of hands and feet, large omphalocele, large diaphragmatic hernia, isolated situs inversus of the liver, renal agenesis, abnormal male genitalia (a thin long penis and rudimentary scrotum) (Fig 1.3) and anal atresia

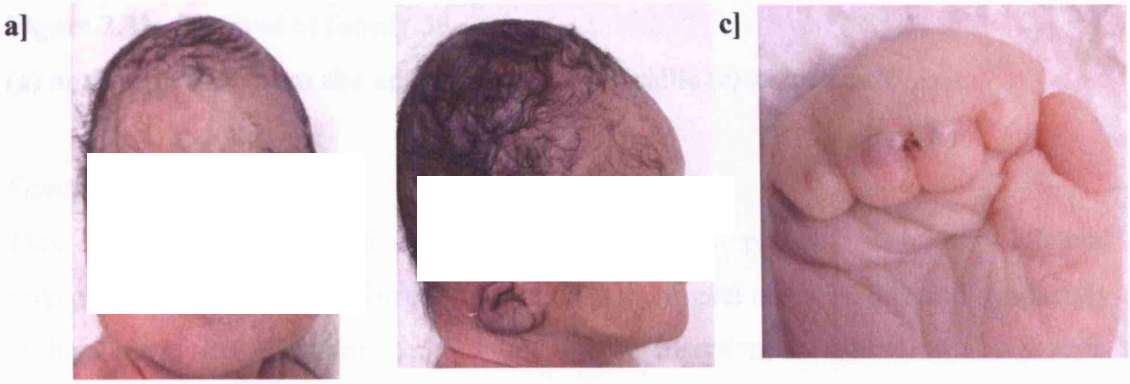

Figure 2.30: Clinical pictures family 37

(a) cleft lip/palate (b) low set ears (c) syndactyly of the right hand

Family 38

This is a non-consanguineous family with one healthy daughter, one daughter that is alive and well in her fifth decade, and a stillborn affected baby. This first affected female (38a) is one of the two patients who were originally described by Fraser (1962) and is now 51 years old (Figure 1.1). She was born with ambiguous genitalia and parents were told that it was a male infant. At the age of one 'he' underwent an inguinal hernia repair and chromosome analysis of a skin biopsy showed a female karyotype (46,XX). She has bilateral complete cryptophthalmos (for which she had an unsuccessful operation), small, simple ears with absent external ear canals, syndactyly of hands and feet, umbilical hernia, ambiguous genitalia and an imperforate anus (which was surgically corrected). She has a conductive hearing loss for which she had an operation at the age of six. The affected still born (38b) had similar abnormalities but severe kidney problems that that were incompatible with life.

a] b] c]

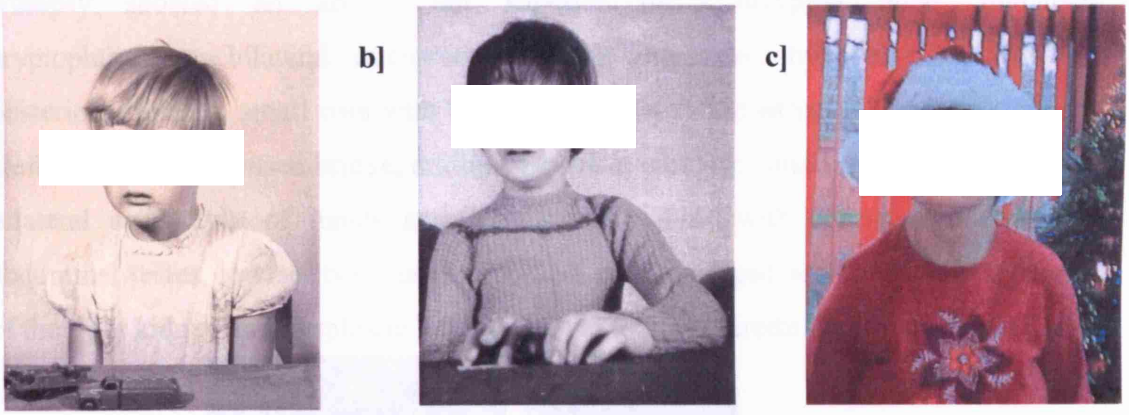

Figure 2.31: Pictures of family 38

(a) at the age of 5 (b) at the age of 7, learning Braille (c) as an adult

Family 39

This is the second child of non-consanguineous parents. She has bilateral cryptophthalmos, small malformed low-set ears, laryngeal stenosis, bilateral syndactyly of hands and feet, low inserted umbilical cord insertion, unilateral renal agenesis, malrotated intestines, cliteromegaly, absent Mullerian duct structures and absent uterus.

Family 40

This is a non-consanguineous family with one previous spontaneous abortion and one termination of pregnancy. The 3rd pregnancy (40a) was terminated at 20 weeks after a prenatal ultrasound showed foetal ascites, hepatosplenomegaly, and al low umbilical cord insertion. Autopsy showed bilateral cryptophthalmos, hypertelorism, low set posteriorly rotated ears, short broad nose with anteverted nares, probably choanal atresia of left nasopharynx, absent nasal wings, tracheal and laryngeal stenosis, bilateral pulmonary dysgenesis (uni-lobular left lung, bi-lobular right lung), bilateral syndactyly of hands and feet, low umbilical cord insertion, vaginal agenesis, unilateral renal and ureteric agenesis, mobile coecum and imperforate anus.

The fourth pregnancy (40b) was terminated at 16 weeks of gestation after a prenatal scan showed congenital malformation suggestive of Fraser syndrome; low umbilical cord insertion, abnormal right kidney, and possible encephalocele.

Autopsy showed an absent left superior parieto-occipital bone, unilateral cryptophthalmos, bilateral microscopic ocular anomalies, hypertelorism, low set posteriorly rotated, small ears with bilateral stenosis of the external ear canals, broad, cleft nose with depressed bridge, midline groove at nasal tip, small mouth, intact palate, bilateral syndactyly of hands and feet, small phallus with absent scrotum, intra abdominal testes, low set two vessel umbilical cord, enlarged adrenal glands, agenesis of the right kidney and dysplastic left kidney, atretic lower urethra and anal atresia

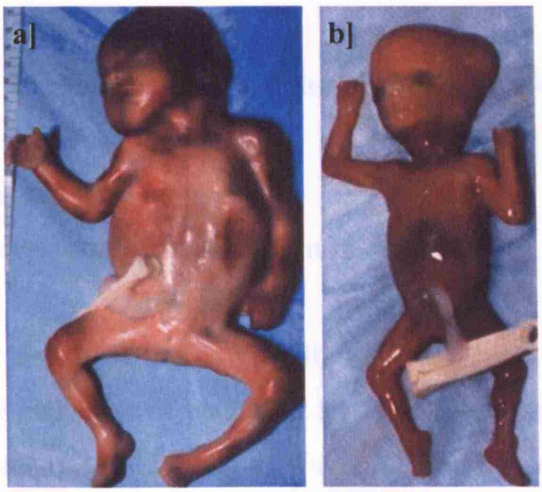

Figure 2.32: Affected foetuses of family 40; (a) Foetus 1 with low set umbilicus and ambiguous genitalia (b) Affected foetus 2 with skull defect.

2.2 Materials

2.2.1 Reagents

All reagents were of AnalaR grade and supplied by Sigma Aldrich or by (British Drug House) BDH Laboratory Supplies, unless otherwise stated. Glassware, solutions and media were autoclaved at 105kPa (15psi), 121°C for 20 minutes as required. Solutions were made using Milli-Q purified water and autoclaved where appropriate.

2.2.2 Non-standard materials

In addition to standard laboratory items, use was made of the following items. Electrophoresis grade agarose was obtained from Gibco-BRL Life technologies. Ultrapure dNTPs were supplied by Bioline 10XMegaBACE™.

DNA electrophoresed on agarose gels was visualised through ethidium bromide fluorescence detected at 300nm on a Chromoto-vue transilliminator. Gels were photographed on a Polaroid Direct Screen Instant Camera using Polaroid film (Polaroid Corporation). PCR amplification and cell cycle sequencing reactions were performed on an Eppendorf Mastercycler gradient thermocycler. DNA sequencing analysis and micro satellite repeat analysis were performed on a MegaBACE 1000 DNA sequencer (Amersham Biosciences).

2.2.2.1 Oligonucleotides:

Oligonucleotides were synthesized to order by either MWG-Biotech or Qiagen.

2.2.2.2 Nucleotide size markers

100 bp DNA ladders were purchased from Invitrogen.

2.2.2.3 PCR amplification and purification products

Qiagen Hotstar® PCR system was used for PCR reactions. In a final volume of 25µl, each reaction contained 0.75 units of Taq DNA polymerase, 10 x buffer (containing Tris-Cl, KCl, $(NH_4)_2SO_4$ and $MgCl_2$), 1.5mM total $MgCl_2$ and 800 µM dNTPs.

For Exo-SAPit reactions Exonuclease from New Englands Biolabs and Shrimp Alkaline Phosphatase from USB Corporation were used.

2.2.2.4 Enzymes

Restriction enzymes were obtained from New England Biolabs or Invitrogen. Enzymes were used with the appropriate buffers, as recommended and supplied by the manufacturers.

2.2.2.5 Commercial kits

MegaBACE ET400R-, MegaBACE ET550R size standards, DYEnamic ET and BigDye® Terminator v3.1 Cycle sequencing kits for MegaBACE were purchased from Amersham Pharmacia Biotech. PCR purification kits were from Qiagen Ltd.
Sephadex® G-50 superfine powder was from Amersham Pharmacia Biotech.

2.2.3 Buffers and solutions

2.2.3.1 General buffers and solutions

TE buffer	10mM Tris-HCl , 1 mM EDTA (pH 8.0)
TAE buffer	40mM Tris-acetate, 1mM EDTA (pH 8.0)

2.2.3.2 Gel loading buffers

Orange G	10X Ficoll-Orange G: 0.25% Orange G, 25% Ficoll, 0.25M EDTA, 50% Glycerol
Genotyping loading mix	7.5µl distilled H_2O, 0.5µl MegaBACETM ET size standard

2.3 Methods

2.3.1 Isolation of DNA

2.3.1.1 Extraction of DNA from paraffin embedded tissue

DNA extraction from post mortem paraffin embedded specimen was required in a few cases, and for this purpose the QIAamp tissue kit (QIAgen Ltd.) was used. This kit allows rapid isolation of genomic DNA from fixed tissue.

In this system, paraffin is removed from the tissue by extraction with xylene, cells are then lysed in the presence of Proteinase K and the DNA is absorbed into a silica membrane by brief centrifugation in a spin column. The salt and pH conditions ensure that protein and other contaminants are not retained on the membrane. The DNA is then washed and eluted from the column membrane. The procedure was done according to manufacturer's protocol.

2.3.2 Quantification of DNA

A 2µl aliquot of DNA was diluted in 198 µl of sterile water and its absorbance measured at 260 nm using a JENWAY 6505 UV spectrometer. The concentration of DNA was then calculated from the A_{260} value (1 A_{260} unit = 50 µg of double stranded DNA).

2.3.3 Amplification of DNA using the Polymerase Chain reaction

2.3.3.1 Selection of primer pairs for genotyping

The primer sequences for amplifying known STS sites were found using Ensembl and Genome database and ordered through either MWG or Qiagen. Microsattelite repeats were amplified by using forward primers labelled with either 6-FAM or TET fluorescent dyes, which emit blue or green light respectively. The primer sequences used for genotyping and their optimal conditions are given in Table 2.2 -2.4.

2.3.3.2 Primer design for direct sequencing

For sequencing of exons, oligonucleotide primers were designed from genomic sequence using the Primer3 programme. Primers were designed to have at least 50-60% GC content, were 20-22 bp in length, had no repeats and were approximately 50bp away from the beginning/ end of the exon. Forward and reverse primers were selected that had similar melting temperatures (TM). The optimal annealing temperature was then determined by performing a PCR reaction on control DNA at a gradient of temperatures. Details of the primer sequences and their optimal determined PCR conditions for direct sequencing are given in Table 2.5 -2.6.

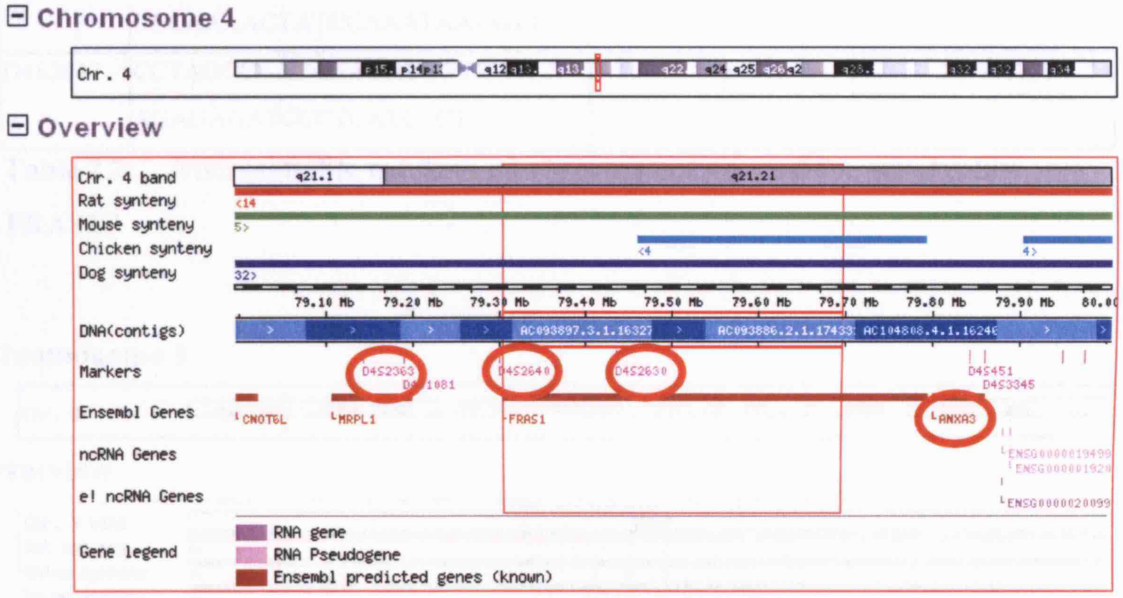

Figure 2.33: *FRAS1* located on chromosome 4q21 with markers highlighted

FRAS1

Marker	Primer sequences forward and (5' to 3')	Annealing Temp. (°C)	Allele size (bp)
D42363	ACCAACTGAATGAGTGCTGG CCATTGCTAAATTCCCATTG	55	121-129
D4S2640	CCTGTCACAGTCCAATGACA GGCCTTGGTAAGGACAGTTA	55	246-257
D4S2630	AAGCCAGGGTTCTACCAGAG TCAGATGAGCCTATTCCCTG	55	202-214
ANX3	ATTTCTAGCATCTTATCTGGTTG CCCAAAACTATCCAAATAAAGTT	55	111-131
D4S2947	CCTAGCCAATAGAGACCGTG AGAGAGATCCCTCATCCCT	55	229-247

Table 2.2: Microsattelite markers on chromosome 4q used for genotyping FRAS1

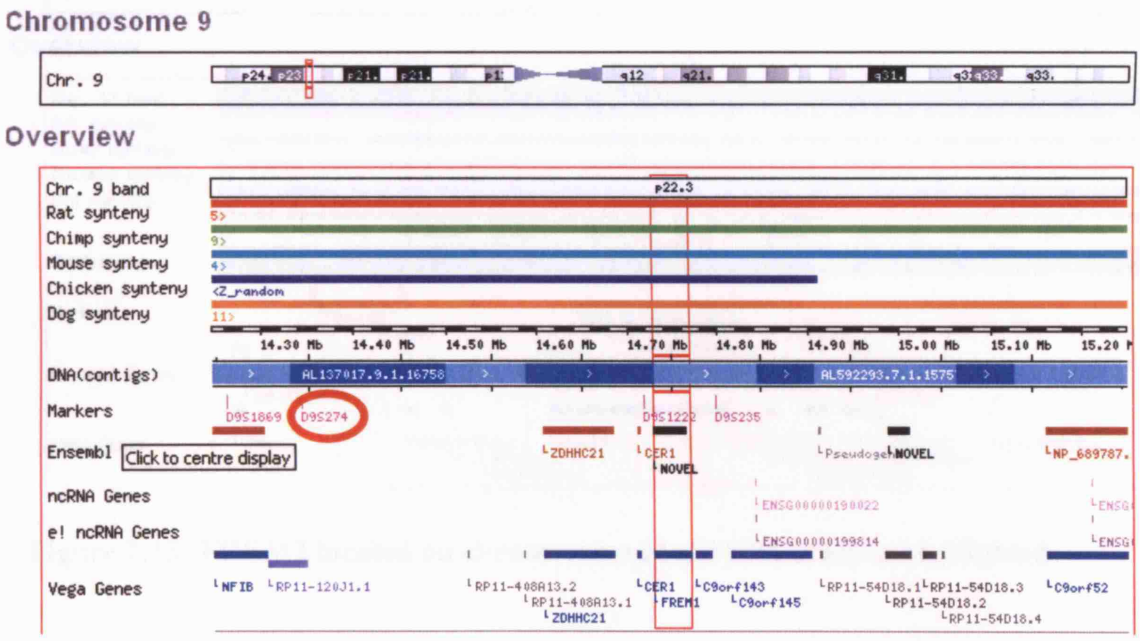

Figure 2.34: FREM1 located on chromosome 9p22 with marker highlighted.

FREM1

Marker	Primer sequences forward and (5' to 3')	Annealing Temp. (°C)	Allele size(bp)
D9S254	TCCTGGGTAATAACTGCCG CACTCACACACACGCTCAG	58	240-270
D9S274	TTCAAGTGATCCTTCACGG TCTAGTTTCTGAGGTGAGCAGTC	58	140-195
D9S285	TGCCAANAGAGTAGATCTGAAG ACCGCAATCAAGCCAAT	58	95-155
D9S156	ATCACTTTTAACTGAGGCGG AGATGGTGGTGAATAGAGGG	58	120-170

Table 2.3: Microsattelite markers on chromosome 9p used for genotyping FREM1

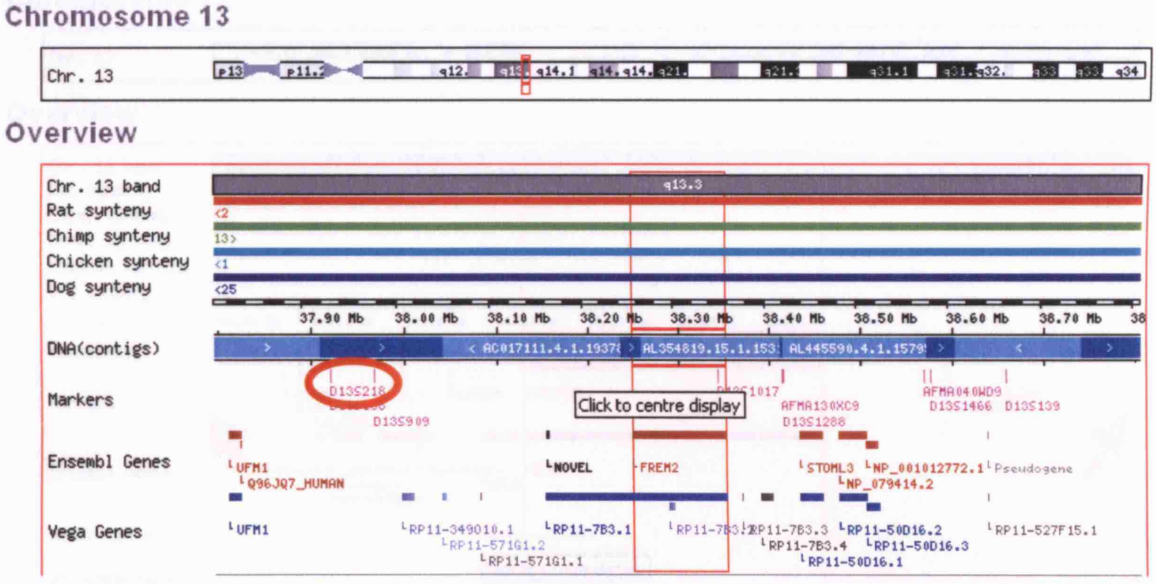

Figure 2.35: FREM2 located on chromosome 13q13 with marker highlighted.

FREM2

Marker	Primer sequences forward and (5' to 3')	Annealing Temp. (°C)	Allele size (bp)
D13S1491	AAGCCACACACAGATGCTAGG CCTCAGCCTCCATAATCTCA	55	128-160
D13S218	GATTTGAAAATGAGCAGTCC GTCGGGCACTACGTTTATCT	55	187-195
D13S1539	GACGTCTCTAGCAATAGGTAAAGGG TCTCTGGACGTAAGGGATGTTTA	55	127-143
D13S1253	CCTGCATTTGTGTACGTGT CAGAGCCGTGGTAGTATATTTTT	55	130-144

Table 2.4: Microsattelite markers on chromosome 13q used for genotyping FREM2

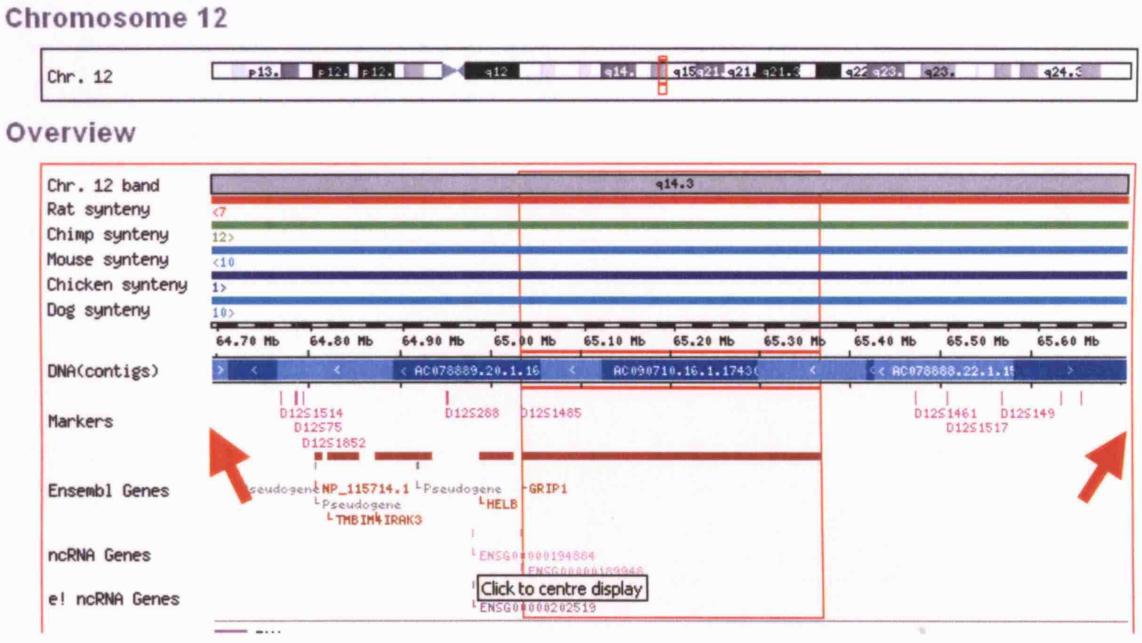

Figure 2.36: GRIP1 located on chromosome 12q14

Markers could not be displayed on the screen (arrows)

GRIP1

Marker	Primer sequences forward and (5' to 3')	Annealing Temp. (°C)	Allele size(bp)
D12S335	TCATCCAGGCTTCACC TGGCAAGGACAGACACA	60	249-265
D12S1668	CTAGGGTCAGAGTTCCTGCT CACTGTCCAATCAAGTAAGGC	57	187-251
D12S1702	AGATGGGTAAAGGGCA AGGTATCTATGAGGGGGTT	57	216-272

Table 2.5: Microsattelite markers on chromosome 12q used for genotyping *GRIP1*

Primer pair	Primer sequences forward and reverse, (5' to 3')	Annealing Temperature (°C)	Product size (bp)
FS1 1f	CCATCTCTTTTCCCCGGAGG	55	296
FS1 1r	CTTCCACCTCACCGAAATACCA		
FS1 2f	GATGCAGTTTTAGCAGCAAGC	58.6	202
FS1 2r	GGGTCAACTCACTCACTCAGG		
FS1 3f	GATGGTGCAGCTTTTGTGTGC	62.5	202
FS1 3r	GATCCAAAAACCTGACCCTTC		
FS1 4f	GTAAGATGACTTAGGGT	53.2	208
FS1 4r	GTTTAACATTCAGCTTTTCCC		
FS1 5f	CCTTTCCCTGAGCTCCATCC	62.5	306
FS1 5r	GGCTGGTTCCCCCGATAGCC		
FS1 6f	GGCTGCACGCCCATGCAGTC	62.5	252
FS1 6r	CCTGCCTGCCATAATCTCAG		
FS1 7f	GGCATGATTTAGTTGGCTGTG	62.5	217
FS1 7r	ATGGTGGCTGGAGTGGGTGG		
FS1 8f	GGTGTCTTATGTGACAGTGC	58.6	213
FS1 8r	CCGGAATAAGAAGTGCATTCC		
FS1 9f	GTCCTCCTGCCTCTGTTGGC	62.5	333
FS1 9r	GTGGACATCCCTGCTGCTGCC		
FS1 10f	CAGTCAGGGAACCCAACTGC	55	215
FS1 10r	GGTAATGTTCCCTCATTAG		
FS1 11f	AAGTTGCATGTTCCTTGG	57.5	183
FS1 11r	CCCCATGATATTTCATGCAG		
FS1 12f	GTTCTTCAGCAGCAAGCTCTC	55	250
FS1 12r	GGGGAAGAGGATCTCTGAGC		
FS1 13f	GGCTTTTTCCTAAGTCCCACAG	55	352
FS1 13r	GCATGACCCCCTCCTTGAC		
FS1 14f	GGGCTGTGTAAGCACTGGC	55	277
FS1 14r	GCTTCCAGCTCCTGGTGTCC		
FS1 15f	GGAACCAACTGACATCCTCC	55	338
FS1 15r	CTCAGCAGCTATTCACAT		
FS1 16f	GAATTTTGCTGAGCAGCCAG	55	333
FS1 16r	CCCATCATACACTCGAGTTCCC		
FS1 17f	GCCCGCGAAGCCCAGCCAGG	55	372
FS1 17r	GCATATAATGTATGGATAGCC		

Primer pair	Primer sequences forward and reverse, (5' to 3')	Annealing Temperature (°C)	Product size (bp)
FS1 18f	GGTGAACTTTTCTAAGTGGG	55	305
FS1 18r	GGCTTTGGTCCCACCTCACAGG		
FS1 19f	CCCAGGAATGTTAATGGGAGAG	55	287
FS1 19r	TAGTCCCCGGTATGACAAGG		
FS1 20f	GATTAGAGTTGGAGGTCTGC	55.9	294
FS1 20r	AAGAGCCCCCTTAAGCCTGG		
FS1 21f	CTTCTGTGCAGTGATGAGAC	58.6	260
FS1 21r	GGTTGATCCTTCCCAGCTAC		
FS1 22f	CAATGTTCTCAGTGTTGGGAC	58.6	296
FS1 22r	GATGGAAGTAAGAACCTCGC		
FS1 23f	CCTATGCAACATCTGTCTAGG	58.6	291
FS1 23r	GATGACTGGTGTTTTCCC		
FS1 24f	CTCCTTGCAGCTGCAGACAG	58.6	229
FS1 24r	GGCTGAGCACAGAGCAGGGC		
FS1 25f	GGCTGTACTGCTGCTGGACA	55	272
FS1 25r	CTGAGAGTCAGCACATGTGAC		
S1 26f	CTGACTCTTCTGGTTGCAAG	60	258
FS1 26r	GTCTGCAAACTCAGCCTTCAC		
FS1 27f	GGAAATTGTAAGGAGGTGGTCTG	60	410
FS1 27r	GAGGCTGGGACAGGGTGGAGG		
FS1 28f	CTAATCTGCCTTGGCCTGAC	60	429
FS1 28r	CCACAGGAAACCTAGATTTCG		
FS1 29f	CTAGTCCACTGGATTGTCC	60	403
FS1 29r	GAAGTTCACATCTAGGTGC		
FS1 30f	CTGAAGCTGTGAATCTCTCTG	60	330
FS1 30r	GCAATCTTGCCACATGGCAC		
FS1 31f	GTAGGAAAGGAGGTAAGGGC	58.6	338
FS1 31r	AATGAACTAACACGTCCCCC		
FS1 32f	GAGGGAAAAACCCACGTAC	55	263
FS1 32r	CCCATCAGCGTGCTGTGTGC		
FS1 29f	CTAGTCCACTGGATTGTCC	60	403
FS1 29r	GAAGTTCACATCTAGGTGC		
FS1 30f	CTGAAGCTGTGAATCTCTCTG	60	330
FS1 30r	GCAATCTTGCCACATGGCAC		

Primer pair	Primer sequences forward and reverse, (5' to 3')	Annealing Temperature (°C)	Product size (bp)
FS1 31f	GTAGGAAAGGAGGTAAGGGC	58.6	338
FS1 31r	AATGAACTAACACGTCCCCC		
FS1 32f	GAGGGAAAAACCCACGTAC	55	263
FS1 32r	CCCATCAGCGTGCTGTGTGC		
FS1 33f	GGTCTGTTTTTGCCTCTGGC	60	232
FS1 33r	CAAGCAGACAAAACTCCAGAG		
FS1 34f	GGCTCTAGTCATTCAGTCAGTG	60	237
FS1 34r	GAGAGTTAGGGCGAGCCTGTC		
FS1 35f	TCCTATCTCTCTCTGATCCC	60	312
FS1 35r	GGCATAAGCCACCATGCCTGGCTC		
FS1 36f	CTGCCTGAAGAGGACCAGAC	58.6	271
FS1 36r	CCATCAAGAGGTTTGAAGAGG		
FS1 37f	ACTCCTAGCCTGGCCTGCGGT	57.5	266
Fs1 37r	GCTTTGCTCTCGTCTGAGACAC		
FS1 38f	TTAGCCTGTAACTCCCTGAG	55	403
FS1 38r	GCATTGCTGCCCAGTCTGCCCC		
FS1 39f	GAGAGAAGCACCCATAGATCT	55	279
FS1 39r	CAGAGCTACAACCAGTTGCTG		
FS1 40f	GATGCTGCTGTCTTACCTGGC	60.4	309
FS1 40r	CTGCCTGGATATTTTGGGGG		
FS1 41f	TCACCCAGGCAGCACTTTGC	60.4	250
FS1 41r	GCAAGGTCCTCCTCCCTCTCC		
FS1 42f	CCACTCTCCCAACTTTATCCTC	58.6	437
FS1 42r	CTACAAGATTCCTGAAGGC		
FS1 43f	TGTTGCCTGGACTTGCTCTC	58.1	509
FS1 43r	CAGAGACACCAATTAACTGGG		
FS1 44f	GAGGCACTTGACATTCTACCC	60.4	433
FS1 44r	CCCACATCACTAACATGGAAG		
FS1 45f	AGTGCCTGGTACCTAGATCC	60.4	376
FS1 45r	GAGTGCACAGTTTATTGCC		
FS1 46f	GTCAAAGCGAGGAAGGTGTG	60.4	295
FS1 46r	GGACTCGAGCCTCGAGGTGA		
FS1 47f	CTGACCTTACTTCTTGGCACC	58.6	302
FS1 47r	TACATGATGTGGCTGCCCTC		

Primer pair	Primer sequences forward and reverse, (5' to 3')	Annealing Temperature (°C)	Product size (bp)
FS1 48f	GAGTATGACACTTCCCTAGCC	58.6	244
FS1 48r	CCAGTCACTCTGCCTACTCCAC		
FS1 49f	AGGACTCACTCTGGGGCTCC	60	366
FS1 49r	TCCTTCAAGGATGCTTGCAC		
FS1 50f	TCAGCAATTGGGCATCACTC	51.6	393
FS1 50r	CCCAAGGTCAATCTACTAGC		
FS1 51f	TACAGCTGTGTAGGCCCTGG	58.1	253
FS1 51r	ACAGGTGAGCTGAACTGGAC		
FS1 52f	TCAGAGGGTCTTGTTCCTGC	57.1	250
FS1 52r	GAAGAATCTGGGAGATAGGC		
FS1 53f	AACTAGGTTTGTTGGTGTGG	*60*	267
FS1 53r	CTTCATCCTGACTCCCTCTGC		
FS1 54f	TGTGTGCTTCCAGCTACAATG	55	486
FS1 54r	GGTTGGGATACCAAAGCTGA		
FS1 55f	GCCCTGGGGAAGCAGAGCAG	57.1	390
FS1 55r	GTGGAAAGTACACATGTGAGT		
FS1 56f	CCAAGCTCATTACCTTACC	51.6	511
FS1 56r	GTGGTATCCCTGAGGAATGG		
FS1 57f	CCACTATTGCAGTGAGGGAG	58.6	311
FS1 57r	CGCCAGGGGAGTTTAGAGAG		
FS1 58f	CCTAGTCAAGGTAAATGGG	58.1	253
FS1 58r	CCAATGTCACATATGTGGACG		
FS1 59f	TTTGGTGGAGCTCACCTGCC	58.1	404
FS1 59r	GCATTGTTCAAGGGTCACCTG		
FS1 60f	ATAGGTGGGTCCCACCCAAG	57.1	426
FS1 60r	CAGAGAATGTTATCAGTAGGC		
FS1 61f	TTTCCTGGGAGTCTGAATCCT	58.1	377
FS1 61r	CCTGCAATCTCAAATGTGTGC		
FS1 62f	GCTGCACTCTCTTGGGTGTG	58.1	328
FS1 62r	CCTGGTTGGGAACCACTGGG		
FS1 63f	GGATCTCTAAAGAAAGGGGGC	60	355
FS1 63r	GCCATGCATGGACCATAAGG		

Primer pair	Primer sequences forward and reverse, (5' to 3')	Annealing Temperature (°C)	Product size (bp)
FS1 64f	GAACCAGATCATGTAAGGAGC	58.6	347
FS1 64r	CCCTTATTGATTGTCCTGGG		
FS1 65f	GGTAGCTCATTGGTGAAGAC	55	264
FS1 65r	CTAGCATGGAAAGACCAGTCC		
FS1 66F	GGTGAGCTCTTCACCCCACC	60.5	324
FS1 66R	GTGCTTCAGGAACAAACAAGG		
FS1 67f	GGGTGAATGAAAATGGAACC	60.5	330
FS1 67r	GGCCCCACTTTGTCTTCGGG		
FS1 68f	TACTTGGAGGTGTCCTTGGG	60.5	301
FS1 68r	GGATGGATGTATGTGCACATGG		
FS1 69f	GGCTTTTGTGGGGCTCTACC	58.6	281
FS1 69r	GCACTATTTCCCAAGCTCCT		
FS1 70f	GAAGGCCATGTCTTTCTGGG	60.5	254
FS1 70r	GCACCATGTTGATTTGGGGG		
FS1 71f	GGGGAAGGGAGGGTGACTC	60.5	284
FS1 71r	TGCCAGTCCGGTGACTTAGC		
FS1 72f	GGGGCACATGGAATCATGG	58.1	380
FS1 72r	GGCTGAAGTCATAGCTGAGG		
FS1 73f	CCCAGTCACTGCCACCTACC	58.1	283
FS1 73r	CCAGAGATGTTGCAGTCCTCC		
FS1 74.1f	GTAGACACCAACCATTGC	57.1	297
FS1 74.1r	GGTGCCATTCTTGACTTGGT		
FS1 74.2f	TCTCAGGGCCCCGGGTCCAG	55	238
FS1 74.2r	CCTGTTGATAAAACAAGCCACC		
FS1 74.3f	CCCTGAATCTGGAGATGCAAG	55	278
FS1 74.3r	GCACCTTTGAACGTGTAAGC		
FS1 74.4f	GCCCGCAGAGGACATTTTGG	55	310
FS1 74.4r	GCTGTCTGCCACTCAAAGC		

Table 2.6: Intronic primers used for *FRAS1* sequencing

Primer pair	Primer sequences forward and reverse, (5' to 3')	Annealing Temperature (°C)	Product size (bp)
FREM2 1f	TGTGAAATCTGGCTGAGTTTTATT	55	600
FREM2 1r	TCTAGACCTTTCCTCATCTCACA		
FREM2 2f	CGGTGGAGAAGCATAAGGAA	55	467
FREM2 2r	CAAAATTCGAGCTTAACACTTCA		
FREM2 3af	CCCCGGCTACAGGAGGAC	55	847
FREM2 3ar	CTGCGGGTAGTGGAGGAGT		
FREM2 3bf	GTGACTCGGAACTTGCCTCT	55	823
FREM2 3br	GCCGAGACTGACCCTCATAG		
FREM2 3cf	GCGCCTCTTTGAACTGGAAT	55	848
FREM2 3cr	GCTGTAGCCCGGACTGAT		
FREM2 3df	TGGCAGCAGCAGGACATA	55	850
FREM2 3dr	GCACCACATGATGTCTGTCA		
FREM2 3ef	GCCACATGAGAGTGTCTGGA	55	848
FREM2 3er	TTCCACCCCTTTATGGACAC		
FREM2 3ff	CCATCCTGCCTGTTGATAGC	55	992
FREM2 3fr	CTCTTGCCCCAAATGGAC		
FREM2 3gf	TTATTCGTTATGGGCCAGGA	55	801
FREM2 3gr	TGCCATCATGTTTGTAGCTGA		
FREM2 3hf	CAGTGGTCACCATCCACAAG	55	810
FREM2 3hr	GCCTATCCTTGCCACTTTCA		
FREM2 4f	TGTCAGCGATTGAATGTATGTG	55	490
FREM2 4r	CCTGTTGCTTGTTTCTTTAGCA		
FREM2 5f	GGCCACATGGCGAGTGC	55	542
FREM2 5r	TCCATTTTCCTGTCAACACATC		
FREM2 6f	TTCCTCCTTTTCCTCCCACTAT	55	448
FREM2 6r	AAGAGGACTCGTCAAAGGTGAA		
FREM2 7f	GCTATTGCAGTTCTAGGGGTGT	55	539
FREM2 7r	GCAGCCAACTTTGAAACCTAAT		
FREM2 8f	GAAGTCCCTAAGAGGAAGCACA	55	371
FREM2 8r	ACAATATACCCAGCCAACTGCT		
FREM2 9f	TGAAACATGGATAAAGTTCAGCAGC	55	545
FREM2 9r	TGGAAGCCCTAGCAAACAAACAA		
FREM2 10f	TTGGCAAGTGACTGAATGGAGA	55	518
FREM2 10r	TGCAGATACCAAAAGGGCATTC		

Primer pair	Primer sequences forward and reverse, (5' to 3')	Annealing Temperature (°C)	Product size (bp)
FREM2 11f	CCCCATACGAATAAGTGCTGGT	55	533
FREM2 11r	CCCCATTTGTTTGTCTTTTTGC		
FREM2 12f	AGCTTTTCCTTCCACACACGTC	55	507
FREM2 12r	AACAAAATTGCACTTGTACCCCTT		
FREM2 13f	CACCCAAATATACTGGACCACA	55	499
FREM2 13r	TCAGTCCTTGAGTTTCGACAAA		
FREM2 14f	GCTCTCCAGGGGCTCTGAGTAT	55	497
FREM2 14r	AATGAACCTTTGTTCGGTGAAGA		
FREM2 15f	ATAAACAGCATGTGGAAATTGG	55	643
FREM2 15r	CAAAGCATTGTGTCTGGCATA		
FREM2 16f	GGCCACTAACTGTCTGCTGTGA	55	474
FREM2 16r	GAAAGAAGTGGAAGGACTTGAAGC		
FREM2 17f	GATTACAGGCATGAGCCACGTC	55	648
FREM2 17r	AGTTGTCTGGTCCCTTGCTGTC		
FREM2 18f	TGCTGTCATTTCCTCTTCTCAATG	55	525
FREM2 18r	CACATATTGCACAAAATTTAAAGCCC		
FREM2 19f	TTCATTTTGGCTCCCTTTTCTG	55	483
FREM2 19r	TTGTGGATTATGAGGTGAAGCTGA		
FREM2 20f	ACAGCCCAGTTTCTTCATCTGC	58	536
FREM2 20r	GGAATCGGATGTCAAGGTCAAA		
FREM2 21f	GCTTTGTCTCTGACTTTGCCGT	58	430
FREM2 21r	AAAGGGCAAATCTCTCCTCATTC		
FREM2 22f	GACTTGATCCCACCAGTTTTGC	58	473
FREM2 22r	ACACCAGCCAGAGTGGGTAAAG		
FREM2 23f	GTAGATGAGGGTGGCAAGGA	58	425
FREM2 23r	CTCCCAAAGTGCTGGGACTA		
FREM2 24f	ACTGCACTCTAGCCTGGGTGAC	60	550
FREM2 24r	ACGCTGGGTTTATGGAGTGCT		
FREM2 25f	AGTGCTTTGCACAGAGCCTTG	58	539
FREM2 25r	GTGAGTAACCCTACCGTGGTGC		

Table 2.7: Intronic primers used for *FREM2* sequencing

2.3.3.3 Genotyping and sequencing PCR

For genotyping and sequencing reactions, the following reaction mix and amplification protocols were used. PCR reactions were carried out in a volume of 15 µl, containing:

- 1 µl of genomic DNA (20-50ng / µl)
- 1.5 µl Biopro ^{TM} Buffer
- 1.5 µl dNTP mix (250 µM each of dATP, dCTP, dGTP, dTTP)
- 0.75 µl MgCl
- 0.1 µl Biopro ^{TM} Taq polymerase
- 9.0 µl sterile H_2O
- 0.5 µl of each primer (10 µM solution)

A negative control was prepared for each reaction; using 1 µl of sterile H_2O instead of DNA, as well as a positive control DNA, using 1 µl of control DNA, known to give the a product of the correct size. The tubes/ plates were sealed and placed in the thermocycler.

For amplification the following protocol was used:

- an initial step for 2 minutes at 94° Celsius, followed by 34 cycles of
- denaturation at 94° Celsius for 20 seconds
- annealing at optimal annealing temperature for 20 seconds
- extension at 72° Celsius for 30 seconds
- a final extension step at 72° Celsius for 7 minutes

2.3.4 Agarose gel electrophoresis

To assess the results of the optimisation experiments, the PCR products were electrophoresed through a 2% agarose gel (2gr agarose, 100 ml 1 X TBE buffer). The solution was heated in a microwave at high power for 1 minute to dissolve the agarose. The molten gel was cooled to ~50 °C under running water before 2µl of a 10 mg/ml ethidium bromide solution was added per 100 ml. The gel was poured into a gel tray of suitable size containing a comb. The gel was left to set for 30 minutes. Once set it was

placed in a tank covered with 1X TBE buffer. The comb was removed and the samples (5 µl of loading buffer plus 3 µl of PCR product) were loaded into the wells and electrophoresed through the agarose at ~100 V. A standard 100 bp latter was used as a size marker. After ~30 minutes the gel was examined under a UV transilluminator. If a single band was present the samples could be prepared for genotyping or sequencing.

2.3.5 Genotyping

2.3.5.1 Preparation of genotyping plate

PCR products were adequately diluted (ranging from 1 in 5 to 1 in 20) in sterile H_2O. Subsequently 2µl of the diluted product was added to the loading mix, containing 7.5µl sterile H_2O and 0.5µl ET-ROX size standards (MegaBACE™ ET400-R or MegaBACE™ ET550R, depending on the size of the products). A polycarbonate 96 well sample plate was filled with the 10µl samples. The size standards enabled accurate sizing of PCR products of up to 550 bp. The samples were then denatured for 2 minutes at 95°C and injected into the MegaBACE™1000 DNA Sequencer following the manufacturer's instructions. Six matrix tubes were taken from storage at 4°C and brought to room temperature. Six 2 ml tubes and a skirted 96 well plate were filled with 1 x linear polyacrylamide (LPA) buffer (Amersham Pharmacia Biotech). The capillaries in the MegaBACE were rinsed with sterile purified water and filled with matrix. Following matrix equilibration and a short pre-run, the capillaries were rinsed again. A pre-injection step was carried out to reduce the salt content of the samples. The 96-sample plate containing the genotyping products was loaded into the cassette and the samples were injected into the MegaBACE at 3000V for 45 seconds. The plate was then replaced into the buffer. The run was started at 10 000V for 75 minutes. Results were analysed using MegaBACE Genetic Profiler software (Amersham Biosciences), which allows automated analysis of di- and tetranucleotide repeats in a Window™ NT format.

2.3.6 Sequencing

2.3.6.1 Purification of the PCR products

For sequencing, PCR samples were purified by ExoSAP-IT. ExoSAP-IT was used to remove excess primers, nucleotides and single stranded DNA.

For each 15µl PCR reaction the following were added:

- 1 µl Exonuclease 1 (10 units)
- 2µl Shrimp Alkaline Phosphatase (2 units)
- 0.5µl 10x Dilatation Buffer
- 1.5µl H_20

This was mixed and incubated in a thermocycler at 37°C for 15 minutes and then at 80°C for a further 15 minutes to inactivate the enzymes. The exonuclease digests any single stranded DNA (such as primers) and the Shrimp Alkaline Phosphatase catalyses the release of 5' phosphate groups from DNA and dNTPs, preventing digested fragments from reannealing.

2.3.6.2 Cycle sequencing

Cycle sequencing reactions were performed using Big Dye (Amersham Biosciences) for the MegaBACE. This uses the dideoxynucleotide chain termination method for sequencing. The dideoxynucleotides are incorporated into the DNA sequence during the annealing step and act as chain terminators by preventing further deoxynucleotides from being incorporated into the product. The dideoxynucleotides are incorporated at different positions along the length of the molecule to be sequenced. Each of the dideoxynucleotides is labelled with a different coloured rhodamine dye and fluorescein. Fluorescein absorbs light energy from the incident laser light and transfers it to the rhodamine dye, which then emits the light at the characteristic wavelength for that dye. This can then be detected and the nucleotide will be identified.

For each reaction, 3 µl of purified PCR product was added to 12 µl of sequencing mix (containing 1µl of forward or reverse primer (5pmol/µl), 2 µl of Big Dye, 2 µl of 5x Seq Buffer, 7 µl of H_2O). The plates were sealed and placed into the thermocycler, for which the following protocol was used:

- initial denaturation step for 2 minutes at 95° Celcius, followed by 34 cycles of
- denaturation at 95° Celsius for 20 seconds
- annealing at 50° Celsius for 10 seconds
- extension at 60° Celcius for 3 minutes

For desalting, 5 µl of water was added to the sequencing reaction to bring up the volume to 20 µl.

2.3.6.3 Sephadex clean-up

Sephadex plates were prepared by adding dry Sephadex powder to 96 well plates and adding 300 µl of sterile water. The plates were then incubated at room temperature for 3 hours to swell the resin and then stored at 4°C. Prior to use, the plates were brought to room temperature and then centrifuged at 910-x g for 5 minutes at room temperature with an alignment frame and collection plate underneath the sephadex plate, to compact the resin. Excess water was discarded. The resin was subsequently washed to remove any salt or ions present, by adding 150µl of sterile water to each well and centrifuged at 910-x g for 5 minutes at room temperature. The water in the collection plate was again discarded. The cycle sequencing products were then added to the centre of each well and centrifuged for 910 x g for 5 minutes at room temperature. The products were collected in a fresh-skirted 96 well plate under the sephadex plate.

2.3.6.4 Running sequencing on the MegaBACE

Prior to sequencing, matrix tubes and buffer tubes need to be prepared. Six matrix tubes were taken from storage at 4°C and brought to room temperature. Six 2 ml tubes and a skirted 96 well plate were filled with 1 x linear polyacrylamide (LPA) buffer (Amersham Pharmacia Biotech). The capillaries in the MegaBACE were rinsed with

sterile purified water and filled with matrix. Following matrix equilibration and a short pre-run, the capillaries were rinsed again. A pre-injection step was carried out to reduce the salt content of the samples. The 96-sample plate containing the sequencing products were loaded into the cassette and the samples were injected into the MegaBACE at 2000V for 60 seconds. Subsequently the run was started at 9 000V for 100 minutes. On completion of the run, Sequence Analyser would automatically assign bases and convert ESD files to ABD files which were then ready for downloading into Sequencher™ 4.1 program for analysis.

2.3.6.5 Sequence analysis

The sequence obtained was analysed using the Sequencher™ 4.1 program (GeneCodes). Sequencing files in ABD format along with the known published sequence for that exon/ region were imported in a Sequencher file. The program aligns similar sequences and highlights discrepancies of bases, facilitating the detection of mutations and SNPs.

2.3.7 Restriction enzyme digestion

For BsrGI digests, to confirm the presence of a mutation, 5µl of PCR product was used with 0.5 units of enzyme and the appropriate buffer and BSA in a 25µl reaction. This was incubated at 37°C for 2 hours. Following digestion, the products were run on a 2% agarose gel.

2.3.8 Bioinformatics

Entrez-PubMed	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi
Primer3-Design	http://www.es.embnet.org/cgi-bin/primer3_www.cgi
NCBI	http://www.ncbi.nlm.nih.gov/
Ensembl human genome server	http://www.ensembl.org/index.html

Chapter 3

Clinical results

Chapter 3 Clinical results

All index patients fulfilled the diagnostic criteria as shown in Table 1.1. There were 27 males (45.8%) and 29 females (49.2%) and three cases (5%) of which the sex was unknown. Consanguinity was present in 25 families (62.5%).

3.1 Pregnancy outcomes

Nineteen cases are still alive and well (32.2%). A prenatal diagnosis was made in 30 cases (50.8%) leading to termination of the pregnancy in 19 cases (32.2%). Two cases were stillborn (3%) and 19 cases died in the neonatal period (32.2%). Of the prenatally diagnosed Fraser Syndrome patients, there are 3 patients that are still alive (1, 21 and 34b). There was a history of one or more spontaneous miscarriages reported in seven families (11.9%)

Investigations and cause of death	Patient no.
Prenatal investigations	1, 6a, 6b, 6c, 6d, 7b, 10a, 11a, 15a, 15b, 15c, 16a, 16b, 17, 19, 21, 22a, 22b, 22c, 24, 25a, 25b, 26, 31, 32b, 33b, 34b, 37, 40a, 40b
Post-mortem investigations	5, 6a, 6b, 6c, 6d, 7b, 10a, 11a, 15a, 15b, 15c, 16a, 16b, 17, 19, 20, 22a, 22b, 22c, 24, 25a, 25b, 26, 31, 32b, 33b, 37, 40a, 40b
Termination of pregnancy	6a, 6b, 6c, 6d, 7b, 10a, 16a, 16b, 17, 19, 22a, 22b, 22c, 26, 31, 32b, 33b, 40a, 40b
Postnatal death	1, 2b, 5, 8, 10b, 11a, 11b, 15a, 15b, 20, 23a, 23b, 24, 25a, 25b, 32a, 34a, 33, 37
Still born	11a, 15c

Table 3.1: Pregnancy outcomes

3.2 Eye abnormalities:

All reported eye abnormalities are listed in Table 3.2

Ocular abnormalities	Patient no.
Complete bilateral cryptophthalmos	6a, 6c, 6d, 7a, 8, 12, 13, 15a, 15c, 23a, 25a, 25b, 26, 32, 36, 37, 38, 39, 40a
Complete unilateral cryptophthalmos	2a, 4a, 4b, 6b, 7b, 9, 10b, 15b, 16b, 18, 20, 21, 23b, 30, 35
Partial bilateral cryptophthalmos	3, 5, 11b, 22a, 24, 27, 28, 39
Partial unilateral cryptophthalmos	2a, 3, 4a, 6b, 7b, 15b, 17, 18, 21, 23b, 29, 35, 40b
Synechiae between cornea and eye lids	11a
Coloboma of upper eyelid	3, 10a, 30
Anophthalmia bilateral	31, 33a
Anophthalmia unilateral	1, 4b, 5, 22b
Microphthalmia bilateral	14, 24, 34a
Microphthalmia unilateral	6a, 16b, 17, 21, 22a, 23b, 26, 30
Malformed globe unilateral	9
Hypertelorism	3, 10a, 17, 25a, 40a, 40b
Broad palpebral fissures	34b
Upslanted palpebral fissures	6c
Abnormal hairline	3, 6b, 7a, 9, 25a, 25b, 26, 33a, 34a, 34b
Ovoid limbus	20
Skin tag under outer angle of one eye	24
Proptosis	25
Aphakia bilateral	28
Absent olphactory nerves	37

Table 3.2: Eye abnormalities

3.2.1 Cryptophthalmos

Cryptophthalmos is defined as complete failure of development of the eyelid folds with continuity of the skin from the forehead to the cheek. The severity of this ocular anomaly is usually not very precisely described in many of the cases, and is listed here as partial cryptophthalmos if documented as such, otherwise it is assumed that it

comprised complete cryptophthalmos, which could often be confirmed by looking at the clinical photographs. Fifty patients (84.7%) are reported to have cryptophthalmos. Complete cryptophthalmos is reported in 34 patients (57.6%), (bilateral in 20 patients (33.9%) and unilateral in 14 patients (23.7%). Partial cryptophthalmos is present in 36.9%, (bilateral in 16.9%, and unilateral in 22% of the cases). There is only one patient that was reported not to have any ocular anomalies (19).

3.2.2 Blepharophimosis

Blepharophimosis is defined as abnormal narrowness of the palpebral fissure in the horizontal direction caused by the lateral displacement of the medial canthi of the eyelids, and was described in 2 patients (7a and 7b). This can sometimes be caused by partial cryptophthalmos. However, it appears from the photographs that patient 7a has bilateral complete and 7b has complete cryptophthalmos of one eye and partial cryptophthalmos of the other eye, so this has been listed as cryprophthalmos.

3.2.3 Anophthalmia

Anophthalmia (congenital absence of all tissues of the eyes) is present in 6 patients (10.2%), bilateral in two (31 and 33a) and unilateral in the other patients. It is an isolated ocular anomaly in 3 patients (1, 22b, and 31) and associated with cryptophthalmos in the other three patients (4b, 5 and 33a).

3.2.4 Microphthalmia

Microphthalmia is reported in 13 patients (22%), Unilateral microphthalmia without any other ocular abnormalities is only reported in one patient (1).

3.2.5 Synechiae

Synechiae between cornea and eyelids is reported in one patient (1.7%).

3.2.6 Coloboma of upper eyelid:

Colobomata of the upper eyelid are reported in 3 patients (5.1%), (unilateral in case 3, bilateral in case 10a, and medial in one case 30). They are reported in combination with

other ocular defects, cryptophthalmos (3), microphthalmia (30), and hypertelorism (10a).

3.2.7 Abnormal hairline

An abnormal hairline is reported in 10 patients (16.9%), being associated with cryptophthalmos in 8 patients, with micropthalmia in 1 patient, and not related to other ocular anomalies in only one case (34b).

3.2.8 Skin tag

A unilateral skin tag under the outer angle of the eye is reported in one patient (1.7%) and is associated with microphthalmia. When looking at the available clinical photographs, patient 21 also seems to have a similar skin tag.

3.2.9 Hypertelorism

Hypertelorism is reported in 6 patients (10.2%)

3.2.10 Proptosis

Proptosis (forward projection or displacement especially of the eyeball) is described in one patient (1.7%) who was also described to have cryptophthalmos, abnormal hairline and hypertelorism.

3.2.11 Aphakia

Bilateral aphakia is described in one patient (1.7%), and seems to be associated with bilateral partial cryptophthalmos.

3.2.12 Absent olphactory nerves

This feature is described in only one patient (1.7%) that also had bilateral partial cryptophthalmos.

3.3 Digital anomalies

All digital abnormalities reported in our patients are listed in Table 3.3. Syndactyly (webbing between the digits of the hands or feet) varies in severity from mild interdigital webbing to a more severe glove-like appearance, and is reported in 56 of our cases (94.9%). A small number of cases had is webbing of the hands (h) or feet (f) only. A bilateral single palmar crease is reported in 3 patients, and is associated with syndactyly in all cases. There is very little information available from patient 14 who is reported to have digital abnormalities of all limbs, so further definition of these anomalies is not possible, but it is most likely to involve syndactyly, since the referrer mentions that the digital anomalies are suggestive of FS. Syndactyly is documented as not being present in only one patient (23b).

Digital anomalies	**Patient no.**
Complete bilateral syndactyly	30
Complete unilateral syndactyly	15c(h)
Partial bilateral syndactyly	3, 4a, 4b, 5, 6a, 6b, 6c, 6d, 7a, 7b, 8, 9h, 10a, 10b(f), 11a(h), 11b(h), 12, 13(h), 15a, 15b, 16a, 16b, 17, 18, 19, 20, 20, 22a(h), 22b, 22c, 23ah, 24, 25a, 25b, 26, 27, 28, 29, 30, 31, 32a(f), 32b, 33a, 34a(f), 34b(f), 35, 37, 38a, 39, 40a, 40b
Partial unilateral syndactyly	1(h), 2a, 2b, 9f, 15c
Single palmar crease	1, 6b, 30
Digital abnormalities of 4 limbs	14
Hypoplastic toenails	3, 6b
Short halluces	5, 34b
Small thumbs	15c
Clinodactyly 5th digits	34a,b
Narrow hands	34a
Broad hands	33b

Table 3.3: Digital abnormalities

3.4 Genital abnormalities

Ambiguous external genitalia are reported in 39 patients (66.1%) and usually comprise cliteromegaly (reported in 10 of the 29 included females (34.4%) and hypoplastic or fused labia (20.6%), and micropenis in 5 of the 27 included males (18.5%) and hypoplastic scrotum in 25.9% of the included males (Table 3.4). Other genital abnormalities in females include partial or complete agenesis of vagina (24.1%) and/ or uterus (10.3%), streak ovaries/ dysgenesis of ovaries (6.9%), common urogenital sinus (6.9%) and absent Müllerian duct structures (3.5%), and in males: cryptorchidism (11.1%), intra-abdominal testes (18.5%) hypospadias (7.4%%), large penis (7.4%), bulbous prepuce (3.7%), and vestigial penile shaft (3.7%). Male genitalia were reported as being normal in only one case (17).

3.5 Affected sib

A positive family history for Fraser syndrome is reported in 35 cases (57.4%).

3.6 Abnormalities of the urinary tract

Renal abnormalities are reported in 78% of our patients, comprising uni- or bilateral agenesis (40.7% and 33.9% respectively), hypo/ dysplasia of the kidneys (15.3%) and cystic kidneys (unilateral in only 1 patient, being associated with renal agenesis in the other kidney) (Table 3.5). Unilateral renal agenesis is associated with agenesis of the ureter in 6 cases (10.2%) and with agenesis or hypoplasia of the bladder in 8 patients (13.6%). Abnormalities of the adrenal glands are only reported in two patients (24 and 40b).

3.7 Abnormalities of the respiratory tract

Laryngeal abnormalities are the most frequently reported respiratory tract anomalies and are reported in 49.2% of our patients. Anomalies include stenosis, hypoplasia or webbing of the larynx. Tracheal abnormalities comprise stenosis, dysplasia and cleft of the trachea (13.6%). Other reported anomalies include, subglottis stenosis (5.1%), abnormalities of the epiglottis (1.7%) and tracheo-oesophageal fistula (1.7%). Both hyperplastic and hypoplastic lungs are reported (5.1% and 8.5% respectively).

Abnormal lung lobulation is reported in 11.9% of the patients. One patient was reported to have a cystic adenomatoid malformation type III of the lung (Table 3.6).

Genital abnormalities	Patient no.
Ambiguous	8, 9, 11a, 12, 15b, 15c, 18, 19, 38
Genital abnormalities	14
Cliteromegaly	4b, 13, 16b, 22b, 23b, 32a, 32b, 34a, 34b, 39
Hypoplastic labia	3, 13, 34a, 34b
Fused labia	1, 23b
Vaginal atresia	6b, 25a, 27, 32a, 34a, 40a
Imperforate vagina with hydrocolpos	15a
Absent uterus	6b, 10a, 39
Streak ovaries	6b
Dysgenesis of ovaries	16b
Absent Mullerian Structures	39
Cloaca?/ Common urogenital sinus	16b, 28
Hypoplastic scrotum	6c, 6d, 20, 24, 26, 37, 40b
Small/ rudimentary penis	6c, 22c, 23a, 31, 40b
Cryptorchidism	23a, 26, 33a
Hypospadias	4a, 20
Large/ long penis	24, 37
Intra-abdominal testes	19, 22a, 26, 33a, 40b
Vestigial penile shaft	6a
Bulbous prepuce	20

Table 3.4: Genital abnormalities

Urinary tract abnormalities	Patient no.
Renal agenesis bilateral	5, 6a, 6b, 6c, 6d, 10a, 10b, 11a 11b, 16a, 16b, 17, 22a, 23a, 23b, 24, 26, 32a, 32b, 37, 38
Renal agenesis unilateral	2a, 4a, 4b, 5, 8, 12, 14, 15a 19, 20, 22b, 25a, 25b, 27, 28, 30, 31, 33b, 34a, 34b, 36, 39, 40a, 40b
Renal hypoplasia bilateral	3
Renal hypoplasia/ dysplasia unilateral	8, 19, 20, 22b, 25b, 29, 31, 40b
Cystic kidneys	31
Agenesis ureter bilateral	6a, 6b, 11a
Agenesis ureter unilateral	8, 32b, 40a
Ureterocele unilateral	4a
Agenesis bladder	6a, 6d, 8, 11a, 16a,
Hypoplasia bladder	16b, 24, 32b
Large bladder	33a, 33b
Agenesis urethra	6a, 11a, 40b
Discoid adrenal glands	24
Enlarged adrenal glands	40b

Table 3.5: Urinary tract abnormalities

Anomalies of the larynx	Patient no.
Laryngeal stenosis	1, 4a, 4b, 5, 6a, 6b, 6c, 6d, 7a, 8, 10a, 10b, 14, 15a, 21, 22c, 24, 25a, 26, 28, 31. 32, 34a, 34b, 39, 40a
Hypoplasia larynx	16b, 22b
Laryngeal web	30
Tracheal cleft	30
Tracheal obstruction	16b, 19, 22, 28, 30, 40a
Dysplastic trachea	16b
Subglottic stenosis	3, 28, 30
Abnormally shaped epiglottis	28
Hypoplasia epiglottis	16b
Tracheo-esophageal fistula	6b
Enlarged lungs	6c
Abnormal lung lobulation	6b, 6c, 16b, 22b, 25a, 40a
Hyperplastic lungs	16b, 19, 22a
Hypoplastic lungs	10a, 10b, 11b, 16a
Cystic adenomatoid malformation III	16b

Table 3.6: Respiratory tract abnormalities

3.8 Malformed ears

Ear abnormalities are reported in 74.6% of our patients, comprising malformed- (16.9%), low set- (40.7%), posteriorly rotated- (10.2%), small- (23.7%), or hypoplastic ears (22.3%). Low set ears are often documented in combination with posteriorly rotated, small or hypo- / dysplastic ears. Partial or complete stenosis of the external ear canal is reported in 15.3% and hearing loss in 8.5% of the cases; being conductive in 3.4% and sensorineural in 5.1%.

Ears:	**Patient no.**
Malformed	1, 3, 13, 15b, 18, 24, 25a, 25b, 32a, 32b, 33a, 39
Low set	4b, 5, 6a, 6b, 6c, 6d, 7a, 10b, 12, 15a, 16b, 18, 19, 20, 22, 26, 27, 31, 34b, 37, 39, 40a, 40b
Posteriorly rotated	6c, 15c, 34a, 34b, 40a, 40b
Small/Microtia	3, 10b, 15b, 15c, 16b, 23a, 23b, 27, 28, 30, 32, 34a, 34b, 39, 40a, 40b
Dys/ hypoplastic	5, 6a, 6d, 8, 10a, 10b, 14, 16b, 17, 20, 26, 30, 34a, 38b
Rudimentary ears	13
Incomplete external meatus unilateral	6b
Hypoplastic external ear canals/ atresia	16b, 23b, 27, 28, 32a, 34a, 37, 38a, 40b
Hearing loss conductive	12, 38
Hearing loss sensorineural	3, 4a, 28

Table 3.7: Ear abnormalities

3.9 Malformed Nose

Fifty-two cases had a documented malformation of the nose. A broad nasal bridge is the most frequently reported nasal abnormality (18.6%). A certain degree of grooved nasal bridge (notched nasal tip, grooved nasal bridge, bifid nose) is reported in 18.6% of the cases. A cleft nose is documented in 3.4%, abnormalities of the nares in 6.8%, and choanal atresia is also reported in 6.8% of the cases.

Abnormal feature of the nose	Patient no.
Abnormal shape	21, 25b
Beaked	24, 35
Hypoplastic/ rudimentary nose/ flat	7a, 14, 15c, 16b, 17, 28
Small nose	10a, 19, 24, 26
Big nose	22
Dysplastic nose	8
Broad nasal bridge	10a, 10b, 16b, 25a, 25b, 26, 34a, 34b, 36, 40a, 40b
Notched nasal tip	4b, 25a, 25b
Grooved nasal bridge	6a, 25a, 25b, 28, 33a, 40b
Cleft nose	1, 40b
Bifid nose/ midline nasal cleavage	22a, 25a, 25b, 28, 40b
Hypoplastic alae nasae	3, 8, 10b, 25a, 25b, 40a
Broad nostrils	6d
Small nostrils	4b
Anteverted nares	40[a]
Choanal atresia	12, 30, 34a, 40a

Table 3.8: Abnormalities of the nose

3.10 Cleft lip/ palate

Cleft lip and/ or palate are reported in five of our 59 reviewed cases (8.5%).

Cleft lip/ palate	Patient no.
Cleft lip and palate	5, 22b, 37
Cleft lip	15c, 17

Table 3.9: Cleft lip/ palate

3.11 Skeletal defects

Skeletal defects comprise skull ossification defects (11.9%), widely spaced cranial bones (3.4%), talipes (6.8%), rib abnormalities (3.4%), shortening of limbs/digits (1.7%), contractures of the limbs (5.1%) clinodactyly of the Vth fingers (1.7%) and abnormalities of the pubic symphysis (1.7%).

Skeletal defects:	Patient no.
Skull defect occipital bones	4b, 30
Skull defect parietal bones	4a
Skull defect temporal bones	6b
Skull defect parieto-occipito bones	40b
Oxycephaly	6d
Widely spaced cranial bones	25a, 32a
Short limbs	32a
Contractures of limbs	5, 23b, 24f
Wide symphysis pubis	5
11 ribs	6a, 6d
Talipes	6c, 17, 22a, 24

Table 3.10: Skeletal defects

3.12 Umbilical hernia

Umbilical hernia or omphalocele is defined as an abnormal protrusion of internal abdominal content into a defect in the umbilical area and is reported in 18.6% of the patients. A low insertion of the umbilical cord is reported in 28.8% and a single umbilical artery in 6.8%.

Umbilical abnormalities	Patient no.
Low insertion umbilicus	5, 6c, 10b, 11a, 11b, 12, 15a, 19, 23b, 28, 32b, 34a, 34b, 35, 39, 40a, 40b
Umbilical hernia	27, 33b, 34a, 34b, 38
Omphalocele	10b, 21, 22a, 22c, 33a, 37
Umbilicus 2 vessels	6c, 14, 33a, 40b

Table 3.11: Umbilical anomalies

3.13 Mental retardation

A developmental delay was reported in only two of our patients. There are however a few reports of other neurological anomalies (Table 3.12)

Brain	Patient no.
Meningo-encephaloceles	2, 7b, 40b
Psychomotor / developmental delay	3, 34a
Microcephaly	3,19
Arnold Chiari mlformation	26
Muscular hypotonia	34a

Table 3.12: Neurological anomalies

3.14 Cardiac defects

Cardiac anomalies were reported in 6 patients (10.2%)

Cardiac defects	Patient no.
Preductal aortic stenosis	6b
Atrial septal defect	15a, 27
Cardiomegaly	25
Persistent ductus arteriosis	25, 33a
Patent foramen ovale	25, 33a
Aneurysm	34b

Table 3.13: Cardiac anomalies

3.15 Abnormalities of the mouth

Abnormalities of the oral region	Patient no.
Small mouth	9, 15b, 34b, 40b
Short philtrum	22
High arched palate	6b, 25, 28
Oral frenula	2a
Tongue tie	28
Bifid uvula	19

Table 3.14: Abnormalities of the mouth

3.16 Gastrointestinal abnormalities

Gastro-intestinal abnormalities were frequently reported in this study. Anal anomalies in particular (stenosis/ atresia and imperforate anus) were reported in 32.2% of the cases.

Gastrointestinal	Patient no.
Diaphragmatic hernia	37
Hepatomegaly	16b
Isolated situs inversus liver	37
Hepatosplenomegaly	40a
Intestinal malrotation	16b, 22c, 39
Duodenal atresia	6d
Non fixation coecum	25a, 40a
Recto-sigmoid narrowing	25[a]
Recto-cervical fistula	16b
Recto-vaginal fistula	12,
Rectal atresia	16b
Anteriorly placed anus	13, 15b
Imperforate anus/ anal stenosis/ atresia	1, 2a, 5, 6c, 6d, 8, 12, 14, 15a, 16b, 17, 22b, 22c, 31, 33a, 34a, 37, 38, 40a, 40b

Table 3.15: Gastrointestinal abnormalities

3.17 Complications during pregnancy

Complications during pregnancy	Patient no.
Foetal ascites	16b, 40a
Nuchal oedema	6d, 16b
IUGR	5, 20, 24
Polyhydramnios	15a
Oligohydramnios	5, 10a, 11a, 16a, 20
Anhydramnios	16b, 17
Placentomegaly	16b

Table 3.16: Complications during pregnancy

3.18 Other reported anomalies

Other dysmorphic features	Patient no
Hoarse cry	4b
Pterygia	6a, 17
Oedematous face	22
Widely spaced nipples	2a, 24, 26
Short thorax	8
Inguinal hernia	2a

Table 3.17: Other reported anomalies

3.19 Cytogenetic abnormalities

Karyotype results were documented in seven cases only, being normal in six cases. A balanced translocation was detected in family 1, who had an affected foetus with the same balanced translocation as her healthy mother; (46,XX) t(2;16)(p15;q22).

Chapter 4

Molecular Results

Chapter 4 Molecular Results

4.1 Genotyping

McGregor had included 22 families in her genotyping analysis. Ten of these families demonstrated convincing evidence of linkage to *FRAS1*. Subsequent molecular analysis of *FRAS1* in these ten families identified mutations in five families (McGregor et al., 2003).

Jadeja had included 11 families (who were unlinked to *FRAS1*) in her *FREM2* genotyping analysis and identified three families with probable linkage to *FREM2*. Subsequent mutation analysis of *FREM2* in these three families identified a common missense mutation (E1972K) in two families (Jadeja et al., 2005)

The families with previously detected *FRAS1* or *FREM2* mutations were only included in the clinical analysis of this project. The consanguineous families that previously demonstrated convincing evidence for linkage to *FRAS1* or *FREM2* but without an identified mutation were re-sequenced for *FRAS1/ FREM2* in the present study.

All other consanguineous families were included in the genotype analysis for the four candidate regions. Three further families who could possibly be distantly related (26, 35 and 36) were also included in the genotyping analysis. Families 35 and 36 were already included in the *FRAS1* genotyping by McGregor.

Some families were only genotyped for two markers. If the markers nearest to the gene showed heterozygosity, then no further markers were tested. The details of the markers are described in Tables 2.2-2.5.

4.1.1 FRAS1

Ten families had a region of homozygosity across the region at chromosome 4q21, containing the FRAS1 gene (Table 4.1).

		FRAS1	(79.3-79.7)	
Marker	**D4S2640**	**D4S2630**	**D4S2947**	**D4S2963**
dist from p tel	**79.3 Mb**	**79.46**	**80.06**	**80.1**
Family				
5	**250**	**?**	**234**	**122**
10*	**253**	**207**	**234**	**254**
11*				
12*		**203**	**233**	
13	**258**	**211**	**231**	**248**
14		**207**	**233**	**248**
15		**202/206**	**233/235**	**252/254**
16	**238**	**203/211**		
17		**207/215**	**233/235**	
18	**253/257**	**211**	**231/235**	**254**
19	**249/253**	**203**	**234/236**	
20	**257**	**207**	**233**	
21		**207**	**236**	**248**
22		**203**	**227/229**	**248**
23*				
24		**202/206**	**227**	
25	**250**	**202/206**	**235**	
26		**207**	**234**	**248**

Table 4.1: Genotyping results for *FRAS1*

*** Evidence of linkage to *FRAS1* according to McGregor (2003)**

Green boxes: fully informative results

Yellow boxes: non-informative results

Orange boxes: Also showing evidence of linkage to *FREM2*

4.1.2 FREM2

Five families had a region of homozygosity across the area at chromosome 13q13.3, containing *FREM2* (Table 4.2). GT still has to be completed in two families.

	FREM2	(38.26-38.36)	
Microsat	**D13S218**	**D13S1288**	**D13S1253**
dist from p tel	**37.9**	**38.4**	**39.05**
Family			
10	**188**	**187**	**136/148**
11	**194**	**183**	**137**
12	**X**	**182/186**	**139**
13	**190**	**186/194**	**138/140**
14	**188**	**187**	**144**
15	**189/193**	**181/183**	**137/139**
16*	**194**		**135/137**
17	**187/195**	**181/197**	**135/141**
18	**190/194**	**183/187**	**135/137**
19	**187/191**	**x**	**137/143**
20	**190**	**x**	**x**
22	**X**	**175/183**	**137/139**
24	**X**	**186/196**	**x**
25	**186**	**182**	**139**
26	**X**	**181/193**	**135/141**
35	**X**	**174**	**136**

Table 4.2: Genotyping results *FREM2*

*** Evidence of linkage to *FREM2* according to Jadeja (2005).**

Green boxed: fully informative results

Yellow boxes: non-informative results

Orange box: Evidence of linkage to both *FRAS1* and *FREM2*

X: failed

4.1.3 FREM1

There are thus far two families that show homozygosity for two markers only across the region at chromosome 9p31. Further SNPs around this area (between D9S274 and D9S285) will be analysed to determine whether there might be evidence of linkage in the four families that show homozygosity for marker D9S274 that lies close to the gene.

		FREM1	14.7	
Microsat	**D9S254**	**D9S274**	**D9S285**	**D9S156**
dist from p tel	**13.05**	**14.3**	**15.5**	**16.2**
Family				
10	**253**	**162**	**112/122**	**147/151?**
12	**253**	**163/165**	**120/128**	**147/151**
13	**256**	**156/164**	**120?**	**132**
14	**261**	**156/164**	**x**	**132**
15	**256/260**	**162**	**110**	**x**
16	**256/260**	**156/162**	**114/126**	**132/149**
17	**257/261**	**164/168**	**112/124**	**132**
18	**261**	**164/168**	**122**	**132/143**
19	**256/260**	**156/162**		**132/143**
20	**253/261**	**162/166**	**106**	**134/153**
22	**257/261**	**162**	**122**	**136/153**
24	**257**	**x**	**x**	**x**
25	**261**	**156**	**112/124**	**134**
26	**261**	**156**	**112/124**	**136/149**
35	**257/261**	**156/168**	**112/124**	**147**
36		**162**	**106/122**	**134/147**

Table 4.3: Genotyping results for *FREM1*

x: failed

Green boxed: fully informative results

4.1.4 GRIP 1

Two families showed evidence of linkage for 2 markers across the *GRIP1* locus on chromosome 12q14. However, further SNPs need to be analysed for the families that show homozygosity for one marker only. Unfortunately, the DNA of family 24 is of poor quality resulting in failure of genotyping results for markers of both *GRIP1* and *FREM1*.

	GRIP1	(65-65.3)	
Microsat	**D12S1686**	**D12S1702**	**D12S335**
dist from ptel	**63.8**	**65.7**	**66.4**
Family			
10	**227/244**	**264/274**	**255/261**
12	**217/246**	**258**	**X**
13	**217/221**	**256/270**	**257/263**
14	**221**	**246/260**	
15	**221**	**261**	**X**
16	**218/245**	**247/258**	**253/265**
17	**242/248**	**260**	**255/265**
18	**217/221**	**260/264**	**255/257**
19	**221/233**	**257/259**	**257/259**
20	**218/228**	**258/264**	**255/265**
22	**221/250**	**258/262**	**255**
24*	**248**	**X**	**259**
25	**221/231**	**264/268**	**257/265**
26	**221**	**260/264**	**259/263**
35	**229/242**	**260**	**257/265**
36	**220**	**246**	**251/255**

Table 4.4: Genotyping results for *GRIP1*

*** not linked to any other candidate gene.**

Green boxed: fully informative results

4.2 *BsrGI* digestion of FREM2 exon 5

Jadeja et al. (2005) identified a mutation in *FREM2* (5914G→ A) in two families of Spanish Gypsy ancestry (Family 8 and Family 9). This disease causing mutation results in a change of charge from an acidic glutamic acid to a basic lysine (E1972K). This residue lies in the CalXβ domain of *FREM2*.

Homozygosity for this mutant allele contains a *BsrGI* restriction site that is cleaved when digested with *BsrGI*, resulting in 2 products (222 and 257 bp).
The wild type allele misses this restriction site and remains uncut after digestion with *BsrGI* resulting in single product of 539 bp.
Since this test is much less time consuming than performing genotyping, a *BsrGI* digestion of exon 5, *FREM2* was undertaken for all families included in our study. Only one patient from another Spanish gypsy family (Family 7) showed the two bands suggestive for the E1972K mutation (Fig 4.1). In all the other families there were no abnormities detected.

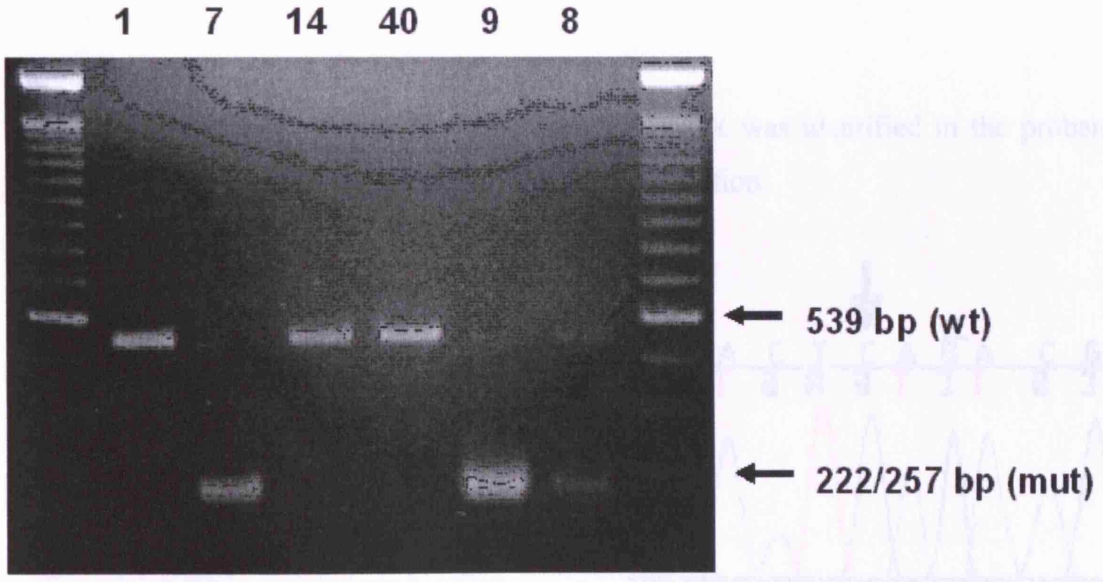

Figure 4.1: Results of BsrGI digestion of FREM2 exon 5 on a 2% gel.

4.3 Sequencing results

Mutation screening was carried out in affected individuals from each of the families that showed evidence for linkage to either the *FRAS1* or *FREM2*. Since preliminary genotype-phenotype results suggested a higher incidence of skull ossification defects and congenital heart anomalies, non-consanguineous families with these clinical features were also included for *FRAS1* sequencing.

4.3.1 *FRAS1*

McGregor (2003) identified nonsense mutations in families 1, 2, 3, 4 and 6. The other consanguineous families that showed evidence of linkage to *FRAS1,* but without an identified *FRAS1* mutations are included for *FRAS1* sequencing in the present study.

Homozygous mutations were identified in the affected individuals of families 5, 11, 20 and 23. Two heterozygous mutations were found in family 40. A single heterozygous mutation was identified in one non-consanguineous family (34). All mutations are shown in the forward- and were verified in the reverse sequencing strand (Fig. 4.2-4.10).

Family 5

A nonsense mutation in exon 74 (11544delC) L3848X was identified in the proband (Fig. 4.2). Both parents were heterozygous for the mutation.

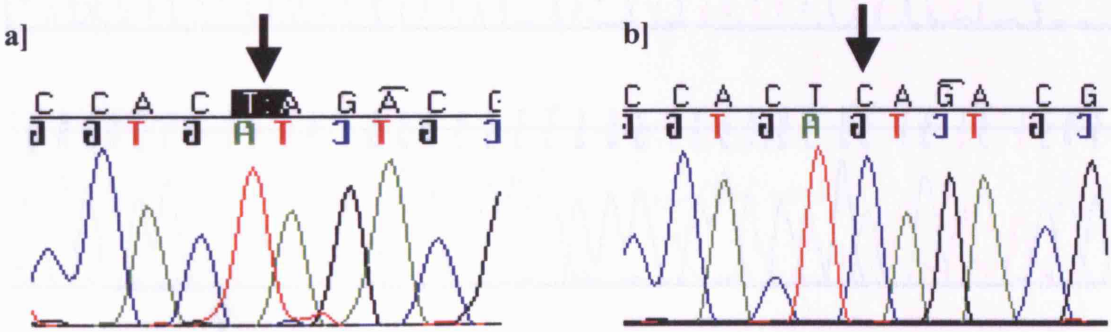

Figure 4.2: Chromatogram of affected patient of family 5 and control
(a)Exon 74: 11544delC in affected patient (b): control DNA

Family 11

A nonsense mutation in exon 63: 9627C→A (Y3209X) was identified in the proband. (Fig.4.3). Both parents were heterozygous for the mutation.

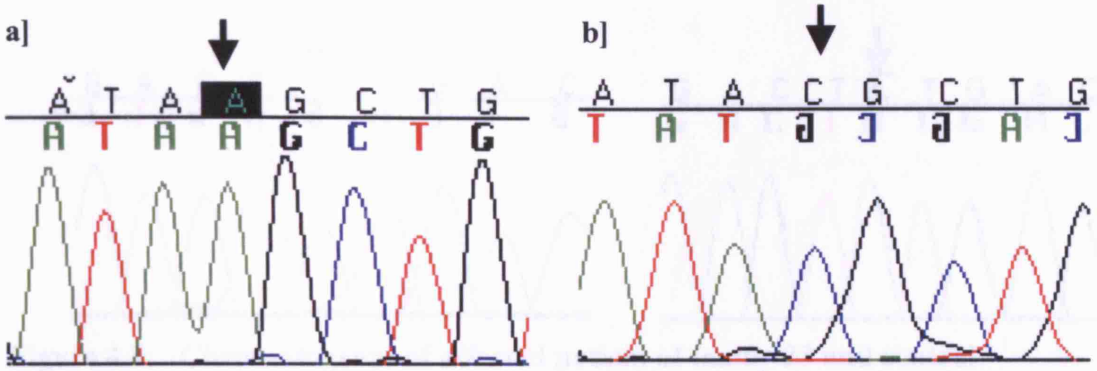

Figure 4.3: Chromatogram of affected patient of family 11 and control

(a) Exon 63: 9627C→A (Y3209X) in affected patient (b): control DNA

Family 20

An insertion of 49 bp was identified in exon 74 of the affected foetus. Both parents were heterozygous for the alteration. (Fig. 4.4)

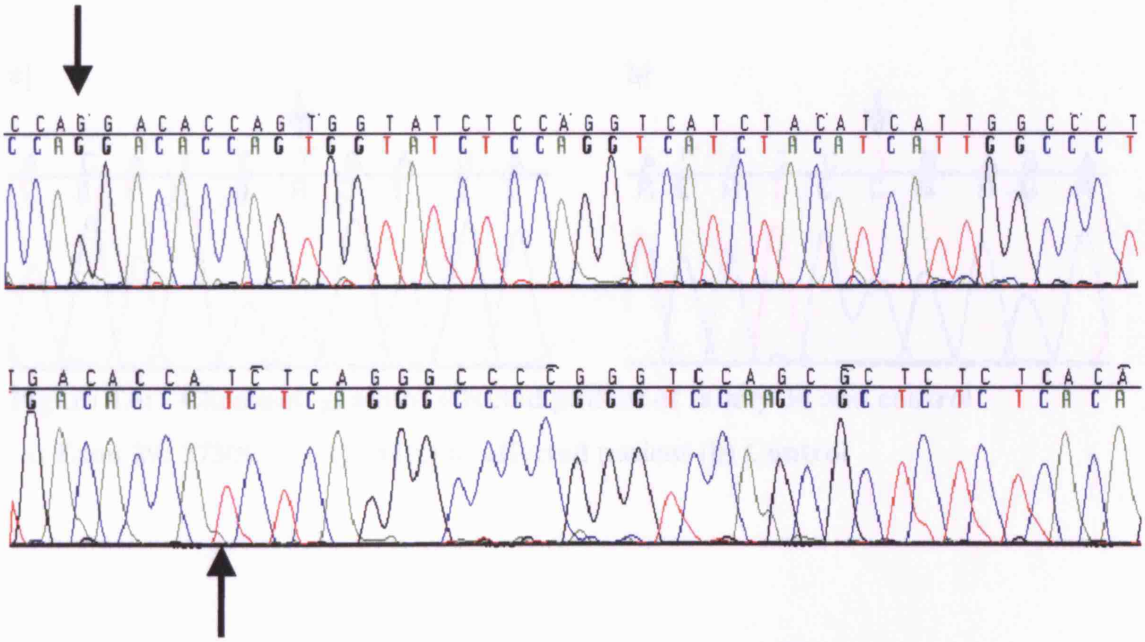

Figure 4.4: Chromatogram of affected patient of family 20

Exon 74: Arrows demarcate the duplication of 49bp at codon 11455

Family 23

A missense mutation in exon 63: 9524A→C (Y3175S), was identified in the proband (Fig. 4.5). Both parents were heterozygous for the mutation.

a] b]

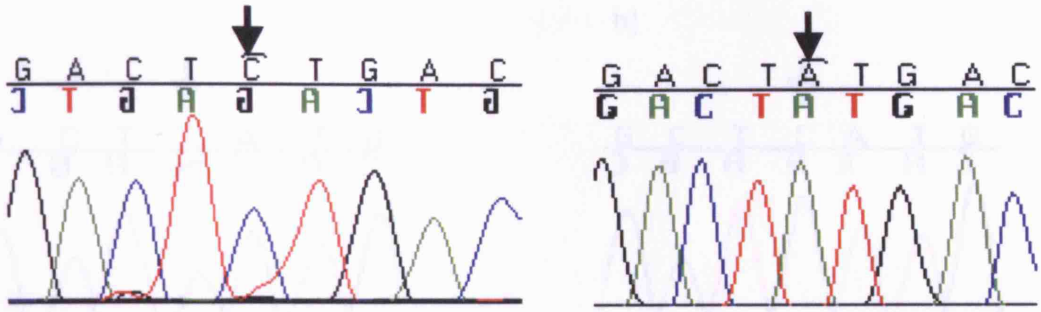

Figure 4.5: Chromatogram of affected patient of family 23 and control

(a) Exon 63: 9524A→C (Y3175S) in affected patient (b) Control

Family 34

A heterozygous mutations in exon 29: 3730C>T (R1244X) was identified in the affected patients (fig 4.6). The same mutation was identified in the mother of the patients.

a] b]

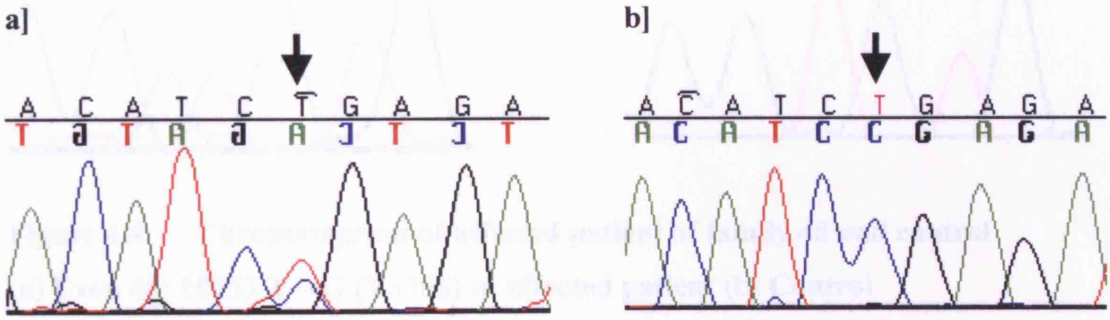

Figure 4.6: Chromatogram of affected patient of family 34 and control

(a) Exon 29: 3730C>T(R1244X) in affected patient (b) Control

Family 40

Two missense mutations: exon 29: 3877C>T (H1293Y) (Fig. 4.7) and exon 65: 10153 T→G (Y3385) (Fig. 4.8) were detected in the proband. Unfortunately DNA from the parents is not available to check if each of them carries one of the mutations.

a] b]

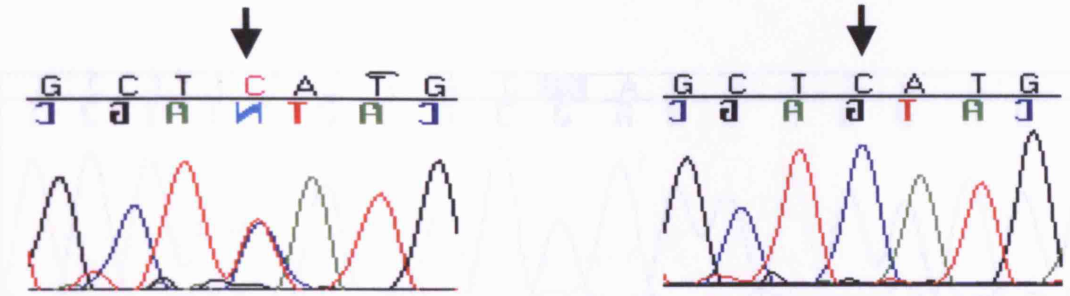

Figure 4.7: Chromatogram of affected patient of family 40 and control
(a) Exon 29: 3877C>T (H1293Y) in affected patient (b) Control

a] b]

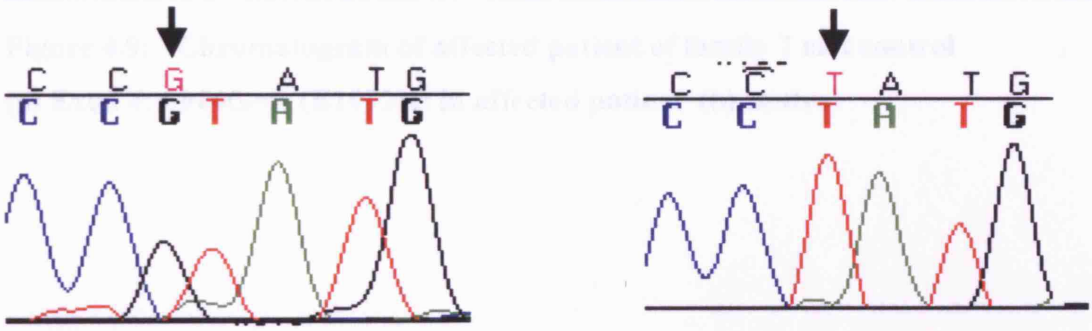

Figure 4.8: Chromatogram of affected patient of family 40 and control
(a) Exon 65: 10153 T→G (Y3385) in affected patient (b) Control

4.3.2 FREM 2

Family 7

Sequencing of exon 5 identified a homozygous missense mutation: 5914G→A, confirming the results of the *BsrGI* digestion of exon 5, *FREM2*.

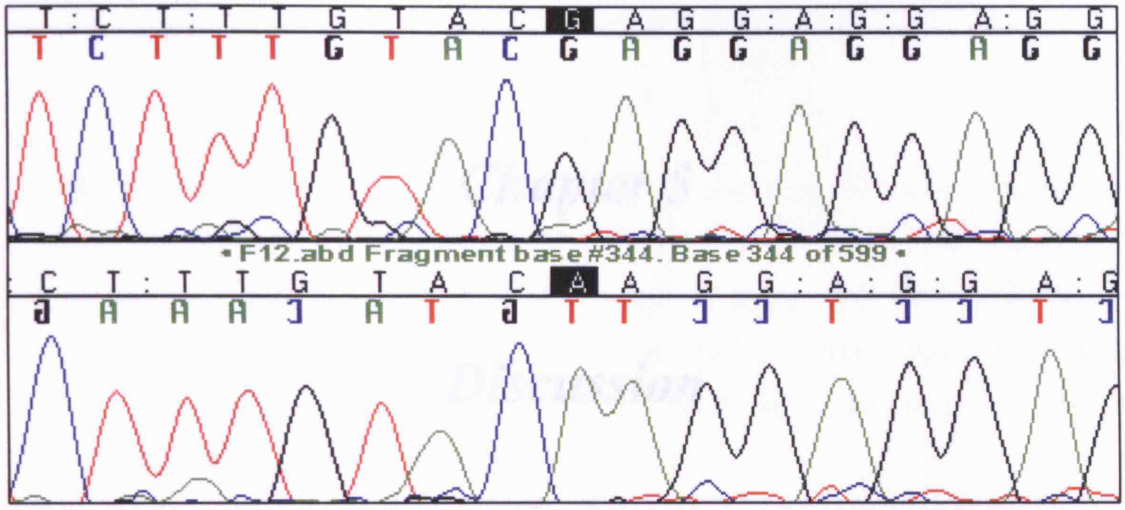

Figure 4.9: Chromatogram of affected patient of family 7 and control

(a) Exon 4: 5914G>A (E1972K) in affected patient (b) control

Chapter 5

Discussion

Chapter 5 Discussion

5.1 Clinical data

Fraser syndrome is a prenatal diagnosis in half of the cases (51%) that were included in this study. The majority of cases (32%) died prenatally after an elective termination of the pregnancy. Three percent of the cases were still born, and 32% died in the neonatal period. At the time of this study 19 patients were still alive (32%).

5.2 Evaluating new diagnostic criteria:

To evaluate the existing diagnostic criteria for FS, we used five different approaches.

(1) We compared clinical data from the present study with data from reviews describing diagnostic criteria. We argued that the most frequently reported abnormalities in the present study group should be features that were used as diagnostic criteria by others (Table 5.1).

(2) We correlated genotype with phenotype. We argued that clinical features of patients with a proven molecular defect might be considered as very reliable indicators for the diagnosis of FS (Table 5.2).

(3) We compared the clinical features of the proband with those of their affected family members. We argued that an ascertainment bias in the relatives will be low and symptoms in relatives show a more reliably frequency of symptoms in FS (Table 5.2).

(4) We compared symptoms in prenatally and postnatally diagnosed cases. We argued that prenatally diagnosed patients may show a more severe phenotype, and that different criteria, depending on the time of diagnosis, may be needed (Table 5.2).

(5) We used the major search machine, the London Dysmorphology Database (LMD), as a tool in determining whether the various combinations of features, used as diagnostic criteria would yield FS as a likely diagnosis, and to which extend combinations of symptoms would differentiate other entities from FS (Table 5.3-5.8).

5.2.1 Comparison with previous literature reviews

These data show higher frequencies of some features than previously reported by Slavotinek and Tift (2002) and most of our results are similar to those published by Thomas et al (1986) (Table 5.1).

Clinical feature	Thomas n=124	Gattuso n=68	Slavotinek n=117	Our study n=59
Cryptophthalmos	85%	93%	88%	85%
Anophthalmia				10%
Microphthalmia				22%
Abnormal hair line		34%	34%	17%
Cutaneous syndactyly	79%	54%	62%	95%
Ambiguous genitalia	80%	49%		66%
Renal agenesis / hypoplasia	84%	37%	45%	80%
Bladder abnormalities		10%	17%	17%
Ear abnormalities	84%	44%	59%	75%
Laryngeal abnormalities	83%	21%	31%	49%
Tracheal abnormalities				14%
Nasal abnormalities	85%	37%		53%
Cleft lip/ palate	11%	7%	11%	9%
Umbilical hernia/omphalocele	28%	12%	6%	20%
Low-set umbilicus		6%	11%	29%
Anal stenosis/ atresia		6%	16%	32%
Skull ossification defect			8%	12%
Rib anomalies				3%
Congenital heart disease		6%	12%	10%

Table 5.1: Comparison of our results with literature reviews.

Conclusion: Our results suggest that especially abnormal position of the umbilicus, skull ossification defects and anal anomalies are more frequently reported in this study than in previous reviews. Renal agenesis and laryngeal abnormalities are much more

frequently reported here than in Slavotinek's (2002) and Gattuso's (1987) study but the present frequencies are still slightly lower than those reported by Thomas (1986).

5.2.1.1 Ascertainment bias in previous studies

Thomas et al. (1986) included only patients in his calculation of whom the presence or absence of a feature had been documented. So every single feature of FS was compared with total group that varied in number depending on the documentation of the feature. This is however unreliable since features can sometimes be missed if the referrer is not aware of it. Slavotinek et al. (2002) did the opposite; she compared an abnormal clinical feature with the complete cohort (n=117), assuming that a feature was not present if it was not documented in the clinical notes. So the frequencies of clinical features as reported by Slavotinek will therefore be lower than in Thomas' series.

Both Thomas and Slavotinek included children in their study who did not fulfil the diagnostic criteria for FS. Thomas included patients with congenital malformations, who might have had another disorder, but belonged to a family where the index case showed classic cryptophthalmos syndrome. This could have resulted in an underestimation of cryptophthalmos in his series. Slavotinek reports frequencies of clinical features in FS, but she also includes patients that proved not to fulfil the diagnostic criteria for this disorder. This results in an over-estimation of some features in FS. It is also notable that many original cases included in their reviews were published in ophthalmic journals that did not include ultrasound reports. This could explain the lower frequencies of renal and laryngeal anomalies in the older literature (Thomas et al., 1986). More recently published FS case reports include prenatal- and post-mortem examination results, resulting in higher frequencies of some diagnostic criteria for Fraser syndrome.

5.2.2 Genotype-phenotype correlations

5.2.2.1 *FRAS1* versus non-*FRAS1*

When analysing the clinical features of the patients with a proven molecular defect, it is notable that patients with a *FRAS1* mutation have more frequently reported abnormalities of the urinary tract; including bilateral renal agenesis/ hypoplasia, bilateral agenesis of the ureters, and agenesis of the bladder. There is also an increase in frequencies of skull ossification defect, umbilical anomalies (abnormal position of umbilicus and hernia/ omphaloceles) and anorectal abnormalities. Cleft lip/ palate is less often reported in the *FRAS1* mutated cases compared to the non- *FRAS1* cases, including cases with *FREM2* mutations (Table 5.2).

It is however too early to present genotype- phenotype correlations since the *FRAS1* screening is not completed yet for all the families that were consistent with linkage and many of the non-consanguineous cases have not yet been included in the *FRAS1* mutation screening. So the non-*FRAS1* group still includes patients that will turn out to have a *FRAS1* mutation in the future.

Conclusion: preliminary data suggest that skull ossification defects and abnormalities of the umbilicus, anus and urinary tract are frequently seen in cases with a *FRAS1* mutation.

5.2.2.2 *FREM2* versus non-*FREM2*

Only slight differences are seen when the clinical features of cases with a *FREM2* mutation are compared with all the other cases (Fig. 5.2). Renal anomalies are less frequently observed in *FREM2* cases than in the non-*FREM2* cases, and skull defects, umbilical abnormalities and heart defects are not seen in the *FREM2* cases. Meningo-encephaloceles are more frequently reported in FS cases with a *FREM2* mutation.

It is however far too early to draw any conclusions from these results since they are based on four cases only. *FREM2* mutation analysis was only performed in those consanguineous families that showed evidence of linkage to *FREM2* and still needs to be completed for a few families. No *FREM2* mutation analysis has been performed yet in the non-consanguineous families. However, normal results of the *BsrGI* digestion of

exon 5 of *FREM2*, in all non-*FREM2* cases, indicates that it is most likely that there are no further cases in this study with the common E1972 mutation in *FREM2*.

Conclusion: cases with a *FREM2* mutation seem to have less severe renal defects compared to all other FS cases, however this study group is however too small to base any conclusions on.

5.2.3 Proband- sib correlation

The diagnosis of FS is easy when cryptophthalmos is associated with other congenital malformations. To make sure that there is no ascertainment bias in this study, we compared the data of the probands with their affected sibs. These results support Gattuso's earlier observations, that the frequencies of anomalies in the affected sibs are comparable with their probands.

Conclusion: there is a strong uniformity of symptoms and a relatively small variability amongst probands and their affected sibs.

5.2.4 Comparing pre- and postnatally diagnosed FS cases.

Since FS is a prenatal diagnosis in half of the cases, it is important to compare pre- and postnatally observed abnormal clinical features. Some major criteria are difficult to detect by prenatal scanning in early pregnancy, or can be normal for gestational age at the time of scanning. The diagnosis of FS is easy when cryptophthalmos is associated with urogenital abnormalities, especially renal agenesis.

Sixty three percent of the prenatally diagnosed FS cases were born after an elective termination of the pregnancy. Bilateral renal agenesis and laryngeal defects are nearly twice as frequently observed in the prenatally diagnosed cases. The more frequently reported lung hypoplasia in the prenatally diagnosed cases is related to the oligohydramnios that is caused by the renal agenesis in these cases.

However, these features cause problems during the pregnancy (oligo-/ polyhydramnios), which result in more frequently performed prenatal scans in the affected pregnancies and earlier detection of these anomalies anyway. Bilateral renal agenesis and laryngeal defects would have resulted in neonatal death if an elective termination had not been performed and it is important that these features are defined as major diagnostic criteria

for the prenatal diagnosis of FS. Apart from these features, there are no other major differences in frequencies of clinical features in pre- and postnatally diagnosed FS cases (Table 5.2). Cryptophthalmos is slightly less frequently observed in prenatally diagnosed cases, which supports the previous results in the literature (Rousseau et al., 2002) and may be related to the difficulties in detecting these anomalies by the use of bi-dimential ultrasound. Our observations were however also based on post-mortem results in 90% of the cases, suggesting that the observed difference in frequencies may be related to the 10% of cases of whom we only received prenatal scanning results.

Conclusion: apart from more frequently reported prenatally detected urinary tract anomalies (agenesis of both kidneys, ureters, and bladder) and laryngeal atresia, no major differences could be identified between prenatally and postnatally diagnosed FS cases.

	FRAS1+ (n=20)	FRAS1- (n=39)	FREM2+ (n=4)	FREM2- (n=55)	Prob (n=42)	Sib (n=17)	Pre (n=29)	Post (n=30)
Cryptophthalmos	81	85	100	84	85	89	77	90
Abnormal hairline	19	15	50	15	18	17	17	17
Cutaneous syndactyly	90	95	100	93	98	94	97	90
Ambiguous genitalia	71	62	50	62	63	78	57	73
Renal agenesis (any)	86	72	25	82	80	72	80	73
- Bilateral renal agenesis	43	31	0	38	33	39	47	23
-Unilateral renal agenesis	43	38	25	42	45	33	33	47
Renal hypoplasia bilateral	4.8	0	0	2	3	0	0	3
Renal hypoplasia unilateral	10	15	25	13	13	17	17	10
Agenesis ureter bilateral	14	0	0	5	5	6	10	0
Bladder abnormalities (any)	19	15	25	15	15	22	27	7
-Agenesis bladder	14	5	25	7	10	6	13	3
-Hypoplasia bladder	0	7	0	5	3	11	10	0
Enlarged adrenal glands	5	0	0	2	0	6	3	0
Laryngeal abnormalities	52	46	50	49	50	50	57	40
Tracheal abnormalities	10	13	0	13	13	11	17	7
Abnormal lung lobulation	14	10	0	13	5	28	23	0
Ear abnormalities	71	74	50	76	78	72	77	70
Nasal abnormalities	43	56	50	53	55	50	60	43
Cleft lip/ palate	5	10	0	9	8	11	13	3
Skull ossification defect	19	8	0	13	10	17	10	13
Umbilical hernia/omphalocele	10	26	0	22	18	28	23	17
Low-set umbilicus	43	21	0	31	25	39	27	30
Umbilicus 2 vessels	10	5	0	7	5	11	7	7
Meningo-encephalocele	10	3	50	4	3	11	7	3
Anal stenosis/ atresia	38	28	25	33	35	28	37	27
Intestinal malrotation	0	8	0	5	3	11	7	3
Congenital heart disease	10	10	0	11	10	11	13	7

Table 5.2: Comparison of frequencies (%) of observed clinical features in FS. (*FRAS1* +: *FRAS1* mutation, *FRAS1-:* no *FRAS1* mutation, *FREM2* +: *FREM2* mutation, FREM2-: no *FREM2* mutation, Prob: probands, sib: siblings, Pre: Prenatally diagnosed, Post: postnatally diagnosed FS cases)

5.2.5 Diagnostic criteria

Features are defined as major criteria when they are present in at least 50% of the cases included in this project and/ or have a high diagnostic specificity (combination of a search criterion results in less than 50 syndromes when entered in the LMD and a low incidence of the feature in the general population). A minor diagnostic criterion can be less specific for FS and occurs more frequently in the general population, so a frequency of at least 10% for a clinical feature in the present study is needed to be defined as a minor diagnostic criterion.

5.2.6 London Medical Data Base (LMD)

To determine the diagnostic specificity of clinical features for FS, each clinical feature was entered as a search criterion in the LMD to see how in how many syndromes the feature could be present. (Table 5.3).

Clinical Feature	(n)	Incidence**
Cryptophthalmos	5	
Syndactyly	89	1:3000
Renal agenesis	105	1:2000
Ambiguous genitalia	59	
Vaginal atresia	39	1:80.000
Hypospadias	215	1:300
Laryngeal/ tracheal anomalies	95	
Anal stenosis/ atresia	132	1:5.000
Dysplastic ears	219	
Depressed nasal bridge*	346	
Umbilical abnormalities*	5	
Skull ossification defects*	57	
Cardiac defects*	150	6-8:1000
Cleft lip	444	1:700

Table 5.3: Number of syndromes (n) that meet a search criterion and incidence

*** Not recognized as a feature of FS, ** According to Moore and Persaud, 2003;**

Based on the combination of data from Table 5.1 and Table 5.3, we suggest that cryptophthalmos, syndactyly of hands and feet, renal agenesis, ambiguous genitalia and laryngeal/ tracheal stenosis should be defined as major diagnostic criteria of FS.
Although abnormal position of the umbilicus and skull ossification defects are also very distinctive features of FS according to Table 5.3, the observed frequencies in our study are less than 50% which makes them less applicable as major criteria so they are defined as minor diagnostic criteria.
To determine whether a clinical feature could indeed be applied as a major diagnostic criterion, a combination of the suggested major diagnostic criteria was entered in the LMD to see how specific the features are for FS. Since ambiguous genitalia were not listed under the clinical features of FS, the entry hypospadias / vaginal atresia was used instead.

5.2.6.1 Combination of three major diagnostic criteria

	A	B	C	D	E
A + B (2)			1	1	1
A + C (12)		1		1	4
A + D (1)		1	1		1
A + E (8)		1	4	1	
B + C (1)	1			1	1
B + D (1)	1		1		1
B + E (1)	1		1	1	
C + D (4)		1			1
C + E (14)	4	1		1	
D + E (3)	1	1	1		

Table 5.4: Number of syndromes (n) that meet a combination of 3 major diagnostic criteria as search criteria.
(A= Syndactyly of hands and / or feet; B= Cryptophthalmos; C= Renal agenesis; D= Ambiguous genitalia (hypospadias in males / vaginal atresia in females); E= Tracheal or laryngeal anomalies)

Conclusion: Most of the combinations result solely in the diagnosis of FS. The combination of syndactyly, renal agenesis and laryngeal or tracheal anomalies however, was slightly less specific and results in three further diagnoses (cutis aplasia-total, Nager acrofacial dysostosis and Pallister Hall syndrome). These entities are however clearly distinctive from FS (Park et al., 1998; McDonald, 1993; Biesecker et al., 1996).

5.2.6.2 Combination of 1major and 2 minor diagnostic criteria

	A	B	C	D	E
a+b (22)	4	1	13	2	3
a+c (5)	3	0	0	4	1
a+d (1)	0	0	0	0	0
a+e (27)	3	0	11	3	9
a+f (10)	0	0	0	0	0
b+c (5)	2	0	0	1	0
b+d (2)	1	0	0	1	0
b+e (36)	3	0	9	1	1
b+f (27)	2	0	1	3	0
c+d (0)	0	0	0	0	0
c+e (4)	3	0	0	3	1
c+f (7)	1	0	0	0	1
d+e (0)	0	0	0	0	0
d+f (14)	0	0	0	0	0
e+f (2)	0	0	0	1	2

Table 5.5: Number of syndromes (n) that meet a combination of 2 minor diagnostic criteria with at least 1 major diagnostic criterion as search criteria.
(a: anal atresia; b: dysplastic ears; c: skull ossification defects; d: abnormal position of the umbilicus; e: congenital cardiac anomalies)

Conclusion: The combination of 2 minor diagnostic criteria with at least 1 major diagnostic criterion results in too many syndromes that meet the criteria. So this combination of criteria is not very helpful in identifying FS as the right diagnosis.

5.2.6.3 Combination of 2major and 2 minor diagnostic criteria

	A+B (n=2)	A+C (n=12)	A+D (n=1)	A+E (n=8)	B+C (n=1)	B+D (n=1)	B+E (n=1)	C+D (n=4)	C+E (n=14)	D+E (n=3)
a+b (22)	1	3	1	1	1	1	1	2	1	2
a+c (5)	0	0	0	1	0	0	0	0	0	1
a+d (1)	0	0	0	0	0	0	0	0	0	0
a+e (27)	0	3	0	1	0	0	0	1	4	2
a+f (10)	0	0	0	0	0	0	0	0	0	0
b+c (5)	0	0	0	0	0	0	0	0	0	0
b+d (2)	0	0	0	0	0	0	0	0	0	0
b+e (36)	0	1	0	1	0	0	0	1	0	0
b+f (27)	0	0	0	0	0	0	0	0	0	0
c+d (0)	0	0	0	0	0	0	0	0	0	0
c+e (4)	0	0	0	1	0	0	0	0	0	1
c+f (7)	0	0	0	0	0	0	0	0	0	0
d+e (0)	0	0	0	0	0	0	0	0	0	0
d+f (2)	0	0	0	0	0	0	0	0	0	0
e+f (14)	0	0	0	0	0	0	0	0	0	0

Table 5.6: Number of syndromes (n) that meet a combination of 2 major and 2 minor diagnostic criteria as search criteria.

Conclusion: The combination of 2 major and 2 minor diagnostic criteria should identify FS in most of the cases. A few combinations results in more than one diagnoses, these can however be easily differentiated from FS.

A+C+a+b : Fraser, Lenz microphthalmia and Towns syndrome

A+C+a+e: Foetal Thalidomide, Lenz microphthalmia and Pallister-Hall syndrome

C+E+a+e: Mohr-Majewski, VATER association, Pallister-Hall and Velo Cardio Facial syndrome

5.2.6.4 Combination of 3 minor diagnostic criteria

	a	b	c	d	e	f
a+b (22)			1	1	8	1
a+c (5)		1		0	0	1
a+d (1)		1	1		0	0
a+e (27)		8	0	0		2
a+f (10)		1	1	0	2	
b+c (5)	1			0	0	2
b+d (2)	1		0		0	0
b+e (36)	8		0	0		2
b+f (27)	1		2	0	2	
c+d (0)	0	0			0	0
c+e (4)	0	0		0		1
c+f (7)	1	2		0	1	
d+e (0)	0	0	0			0
d+f (2)	0	1	0		0	
e+f (14)	2	2	1	0		

Table 5.7: Number of syndromes (n) that meet a combination of 3 minor diagnostic criteria as search criteria.

Conclusion: The combination of 3 minor diagnostic criteria should identify FS in most of the cases. So in combination with at least one major diagnostic criterion, it should be easy to make the diagnosis. Congenital cardiac anomalies however, are observed in many different syndromes. If congenital cardiac anomalies are part of the three minor diagnostic criteria then the combination results in eight differential diagnoses (Table 5.7). These diagnoses (Antley Bixler, Baller-Gerold, Duane anomaly -radial defects, FG syndrome, Hydrocephaly with features of VATER, Lenz microphthalmia, Maternal diabetes syndrome, and Treacher Collins syndrome) are however clearly distinctive from FS (Al Baradie et al., 2002; Dixon, 1995; Iafolla et al., 1991; Loffredo et al., 2001; Temtamy et al., 2000; Temtamy et al., 2003; Zwamborn-Hanssen et al., 1995).

The combination of these three minor diagnostic criteria (a+b+e) with renal agenesis as a major diagnostic criterion only slightly narrows the differential diagnoses. Apart from FG syndrome and Treacher Collins syndrome the differential diagnoses remains the same as mentioned for a+b+e.

5.2.6.5 Combination of one major and three minor diagnostic criteria

	A	B	C	D	E
a+b+e (8)	1	0	6	1	0

Table 5.8: Number of syndromes (n) that meet a combination of 3 minor and at least 1 major diagnostic criterion as search criteria.

5.3 Revised Diagnostic Criteria for Fraser syndrome

Based on the results of this study, we suggest that the diagnostic criteria of Thomas et al. (1986) should be revised according to Table 5.9. The diagnosis can now be made if 3 major criteria, 2 major and 2 minor or 1 major and 3 minor criteria are present in a patient. Unfortunately, not all of the suggested minor criteria (skull ossification defects, umbilical position abnormalities, congenital heart defect) are listed as a clinical feature observed in FS in the LMD.

Major Criteria	Minor criteria
Syndactyly	Anorectal defects
Cryptophthalmos spectrum	Dysplastic ears
Urinary tract abnormalities	Nasal anomalies
Ambiguous genitalia	Skull ossification defects
Laryngeal and tracheal anomalies	Umbilical abnormalities
Positive family history	Cardiac abnormalities

Table 5.9: New diagnostic criteria for Fraser syndrome

A few of the minor criteria as defined by Thomas et al. (1986) are revised to major diagnostic criteria (renal agenesis and laryncheal/ tracheal abnormalities). There are a

few new minor diagnostic criteria (anorectal defects, skull ossification and cardiac defects).

The ear anomalies are mainly described as dysplastic ears and the most frequently reported nasal abnormality is a wide nasal bridge. The latter is however not recognized as a feature of FS. Although they are very common in FS, these features are also very common in other syndromes and are thus defined as minor diagnostic criteria for FS. Mental retardation, cleft lip/ palate and skeletal defects have been removed from the list of diagnostic criteria since they were reported in less than 10% of the patients included in this study. These features will now be listed under 'additional observed anomalies in Fraser syndrome' (Table 5.10).

Mouth abnormalities	**Gastro intestinal**
Small mouth	Diaphragmatic hernia
Short philtrum	Hepatomegaly
High arched palate	Isolated situs inversus liver
Oral frenula	Hepatosplenomegaly
Tongue tie	Intestinal malrotation
Bifid uvula	Duodenal atresia
Cleft lip/ palate	Non fixation cecum
Teeth abnormalities	Rectosigmoid narrowing
	Rectocervical fistula
Respiratory tract	Rectovaginal fistula
Subglottic stenosis	Rectal atresia
Abnormal epiglottis	Anteriorly placed anus
Enlarged lungs	
Abnormal lung lobulation	**Skeletal defects**
Hyperplastic lungs	Rib anomalies
Hypoplastic lungs	Short limbs
Cystic adenomatoid malformation type III	Wide symphysis pubis
Digital anomalies	**Other dysmorphic features**
Single palmar crease	Micrognathia
Hypoplasia toenails	Oedematous face
Short halluces	Widely spaced nipples
Small thumbs	Short thorax
Clinodactyly 5th digits	Hypoplasia thymus
Narrow hands	Absent thyroid cartilage
	Tracho-oesophageal fistula
Neuromuscular abnormalities	Inguinal hernia
Meningo-encephaloceles	
Encephalocele	**Pregnancy complications**
Psychomotor / developmental delay	Foetal ascites
Microcephaly	Nuchal edema
Arnold-Chiari malformation	Placentomegaly
Muscular hypotonia	

Table 5.10: Additional observed malformations in FS

5.3.1 Digital anomalies

Syndactyly of the skin is present in nearly all our cases (90.2%) and is even more frequently reported than cryptophthalmos.

All patients documented with skeletal defects of the limbs such as arthrogryposis and clubfeet also had bilateral renal agenesis. So these features are part of the Potter sequence (secondary to the oligohydamnios that is caused by bilateral renal agenesis), and should be interpreted as such instead of being a specific feature of FS.

5.3.2 Cryptophthalmos spectrum

Cryptophthalmos, defined as complete failure of development of the eyelid folds with continuity of the skin from the forehead to the cheek, is present in 50 of our 59 patients (85%). The severity of cryptophthalmos varies from complete bilateral to incomplete, unilateral cryptophthalmos or just colobomata, or synechiae of the eyelids to the cornea. Complete cryptophthalmos is often associated with complete absence of eyebrows and eyelashes. The incomplete or partial form involves medial defects of the eyelids and eyebrows only, whereas the abortive form (colobomata) is defined as "replacement of the superior eyelids by an epidermal fold that adheres to the upper third of the cornea with normal inferior eyelids" (Francois, 1965). An abnormal frontal hairline (tongue of hair extending from the lateral forehead to the lateral eye is reported in ten cases (16.9%). However, secondary hair only becomes longer than lanuga after 28 weeks of gestation, which means that an abnormal hair pattern could not have been recognized before that time (England, 1983). Thirty percent of the affected pregnancies in our study were terminated in the first or second trimester, which means that an abnormal hairpattern is actually present in 8/39 (21%) of the cases where it could have been recognized. It is nearly always associated with other ocular anomalies (cryptophthalmos and microphthalmia) in these cases, so it may well be an indicator for maldevelopment in the region of the upper eyelid fold. This has already been suggested by Roizenblatt, who described a patient with frontonasal dysplasia with an abnormal hairline implantation in the corresponding frontal region (Roizenblatt et al., 1982). The authors proposed that the eyelid is responsible for the area of hair growth suppression around the eyes (Fig 5.1).

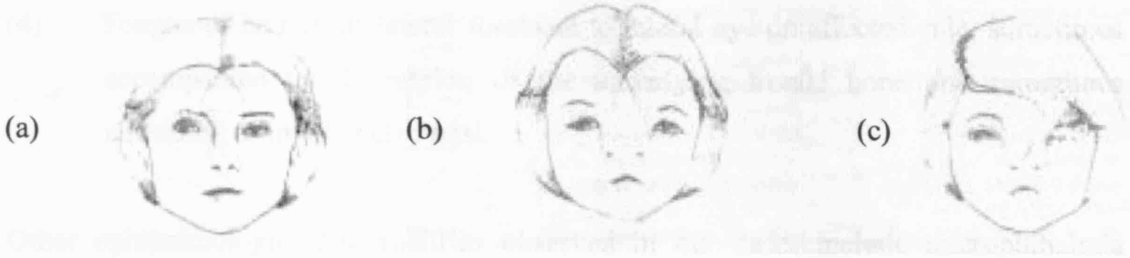

Figure 5.1: Eyelid is responsible for an area of hair growth suppression
(a) Facial aspect of a normal person: dashed lines in the frontal region delimit the areas under the suppressive influence of the superior eyelids. (b) ocular hypertelorism. Greater space between the eyes creates conditions for a tongue shaped area in the mid-frontal region, not to be under the suppressive influence of the eyelids. (c) Presence of upper eyelid in the right eye ensures a suppressive activity around this eye. The absence of a superior eyelid in the left eye, would allow hair to grow in an area normally devoid of it. (From Roizenblatt et al., J Pediatr Ophthalmol Strabismus, 1982)

We therefore suggest that a cryptophthalmos spectrum should be regarded as a main diagnostic criterion for Fraser syndrome, consisting of the following possibilities:

(1) Complete form: bi- or unilateral replacement of the eyelid fold by skin that extends from the forehead to the cheek.

(2) Incomplete form (medial defects of the eyelids and eyebrows only) characterized by involvement of nasal orbital area alone.

(3) Abortive form: replacement of the superior eyelids by an epidermal fold that adheres to the upper third of the cornea with normal inferior eyelids (Francois, 1965). This is most likely to be caused by failure of the upper eyelid to develop and not being able to reach the lower lid and its margins to fuse with, resulting in congenital symblepharon with a normal lower lid and relatively normal globe. This is very similar to congenital coloboma of the upper eyelid and microblepharon, conditions also attributing to anomalous development of lid folds.

(4) Tongue of hair from lateral forehead to lateral eye on affected side, sometimes accompanied by depression of the underlying frontal bone and sometimes extending to involve alae nasi.

Other ophthalmologic abnormalities observed in our cases include microphthalmia (22%), anophthalmia (10.2%), hypertelorism (10.2%) and defects of the anterior segment of the eye. The documentation of these features seems to be related to the interpretation of the referring physician and it is not excluded that they might be associated with the milder variant of the cryptophtalmos spectrum in a few cases (34a and 34b). There is only one patient included in this study, who is reported to have no ocular anomalies (19).

Blepharophimosis is reported in two patients belonging to family 7 but should be regarded as a mislabeling by the referrer since both patients definitely had cryptophthalmos (Figure 2.7). This illustrates the importance of using the same jargon amongst referrers to avoid misinterpretation and under-documentation of certain clinical features.

5.3.3 Genital anomalies

Ambiguous genitalia, including cliteromegaly, hypoplastic/ fused labia, and vaginal atresia in females and hypoplastic scrotum and small/ rudimentary penis in males, are the most frequently reported genital abnormalities and comprise 39 of our 59 cases (66.1%).

However, hypoplastic scrotal sacs are reported in 7 cases of which 4 involved foetuses that were born after a terminated pregnancy in the first or second trimester. The same accounts for 3 out of the 5 cases that were reported to have intra-abdominal testes. Since these features could be normal for the gestational age, caution should be taken in the interpretation of these reported 'abnormalities'.

5.3.4 Anomalies of the urinary tract

Renal abnormalities are frequently described in Fraser syndrome. Agenesis or dysgenesis of the kidneys is reported in 79.7% of our cases. Many of these cases also

had lower urinary tract anomalies (a-/ dysgenesis of the ureters, bladder and urethra). Forty-five of our 59 patients (76.3%) had renal agenesis (21 bilateral and 24 unilateral); nine cases were reported to have renal hypoplasia or dysplasia (1 bilateral and 8 unilateral). The cases reported with unilateral renal hypoplasia often had agenesis of the other kidney. Cystic dysplasia of the kidneys was reported in only one patient, being unilateral and associated with agenesis of the other kidney. Agenesis or hypoplasia of the bladder was reported more frequently than agenesis of the ureters (in eight and six cases respectively). This illustrates the incomplete documentation of the ureters in those cases where agenesis of the kidneys was associated with agenesis of the bladder. Our data illustrate that abnormalities of the urinary tract, and agenesis of the kidneys in particular, are much more frequently present in Fraser syndrome patients than previously thought. This can be explained by the more regularly performed prenatal- and post-mortem investigations of our patients. Earlier reports of Fraser syndrome cases with renal defects and syndactyly but without eye abnormalities already suggested that renal agenesis should be regarded as a major diagnostic criterion for FS (Burn and Marwood, 1982; Codere et al., 1981; Lurie and Cherstvoy, 1984; Mortimer et al., 1985). This criterion is also very helpful in making a prenatal diagnosis of this disorder (Vanlieferinghen et al., 1989). Ureter /bladder and internal genital abnormalities are often associated with the renal defects, suggesting a possible underlying defect in a common pathway.

Case 24 illustrates the prenatal diagnostic pitfalls in cases of renal agenesis; prenatal scans had shown an abnormal (horse shoe) right kidney whereas autopsy showed renal agenesis of the right kidney and enlarged adrenal glands, the latter being misinterpreted for kidneys during the prenatal investigations.

5.3.5 Affected Sib

Affected family members are reported in 59.3% of our cases. This is a higher frequency than mentioned in previous studies and can be explained by an ascertainment bias in our series; we were keener to include patients in the molecular study that had either a positive family history or consanguineous parents, than isolated cases only. Thomas et al., (1986) suggested that an affected sibling should be one of the major criteria to fulfil

for the diagnosis of Fraser syndrome. However, in the same paper he proposed that in the presence of a previous affected sibling, ''the diagnosis could be made without fulfilling the criteria''. Now that mutation analysis is available for FS, a clear description of the "affected sib" is necessary. A positive family history is counted as a major diagnostic criterion in the presence of an affected family member who meets the diagnostic criteria, or when a mutation in one of the *FRAS/ FREM* genes has been identified in an affected family member.

5.3.6 Abnormalities of the respiratory tract

Stenosis and hypoplasia of the larynx (49.2%) and trachea (13.6%) are the most commonly reported abnormalities of the respiratory tract in the patients included in this study. The higher incidence of laryngeal stenosis in this study compared with data from previous reviews can probably be explained by the more frequently undertaken autopsy or radiological investigations in our series, which seemed not to be included in many reports in the ophthalmologic literature. The documented lung abnormalities (enlarged, hyper- and hypoplastic lungs) are secondary to either laryngeal stenosis (hyperplasia) or renal agenesis (lung hypoplasia due to oligohydramnios) in the majority of cases. Cystic adenomatoid malformation type III is reported only once and has not been reported before in FS. This anomaly was reported in combination with very detailed information of other respiratory tract anomalies, which could possibly indicate that this anomaly might have been overlooked in other patients. Abnormal lung lobulation however has been reported in 11% of our cases and is possibly primarily caused by the underlying *FRAS/FREM* mutation, since *bl* mice are also described with lung lobulation defects (Petrou et al., 2005). Lung lobulation defects have thus far only been looked at in the *bl* blebbing mutant, so it is too early to suggest a phenotype- genotype correlation for this feature.

5.3.7 Abnormalities of the ears

Karas made a subdivision of ear abnormalities that can be observed in FS, occurring either isolated or in combination with the other ear defects; (1) low set ears (2) stenosis

or atresia of the external auditory ear canal, (3) microtia, and (4) skin of the superior helix fused to the scalp (Karas and Respler, 1995).

These four subdivisions are all reported in our study and do not differ significantly from previous studies. Although the description of dysmorphic ears can vary among referrers, the most common reported anomalies in our series comprise low set (40.7%), malformed (16.9%), posteriorly rotated (10.2%) and/ or dysplastic ears (22.3%). Stenosis of the external ear canal is reported in 15.3%, microtia in 28.8% and fusion of the skin of the superior helix to the scalp in only 1.7%, however the latter may have been documented as 'malformed ear' in general. Abnormal ears are more frequently reported in our patients than in Slavotinek's or Gattuso's study and are comparable to Thomas' results (Gattuso et al., 1987; Slavotinek and Tifft, 2002; Thomas et al., 1986). Half of our cases were diagnosed prenatally and the diagnosis was confirmed after an elective termination of the pregnancy in the first or second trimester. Abnormal ears as observed in FS are however difficult to interpret at this stage of gestation, since low set ears and dysplastic ears could be normal for gestational age till approximately 32 weeks (Moore and Persaud, 2003).

Atresia of the external ear canals can be a result of a defect in the recanalisation of the meatal plug, resulting from a failure in apoptosis possibly related to the underlying mutation in one of the *FRAS/FREM* genes. This can either occur together or separate from the bleb formation around this area, which could also result in an occlusion of the external ear canal. However, the unilaterality of the observed external ear canal defect, favours the "bleb-formation theory" in that area.

5.3.8 Malformations of the nose

The nasal abnormalities are reported in 52% of our cases and are similar to those mentioned in the previous review articles. The exact description of the anomalies however still depends on the dysmorphological experience of the referrer. A broad nasal bridge, grooved nasal bridge, midline cleavage and hypoplastic alae nasi are frequently reported nasal abnormalities. It is most likely that bleb formation in this area prevents the normal development of the nose, leading to a flat or hypoplastic nose.

5.3.9 Cleft lip/ cleft palate

The incidence of a cleft lip and/ or palate (8.5%) in our patients is slightly lower than previously reported. We agree with Slavotinek that this feature is not a very helpful diagnostic criterion; however it might still be associated with FS, so this feature will also be listed under the 'additional observed anomalies in FS' (Table 5.10).

5.3.10 Skeletal defects

Skull ossification defects were the most frequently reported skeletal anomalies (11.9% of our cases). Although there are a few case reports describing this feature in FS patients (Burn and Marwood, 1982; Ramsing et al., 1990; Serville et al., 1989), this frequency is higher than previously reported by Slavitinek. Since it is a distinctive feature and reported in more than 10% of our cases, it is will be defined as a minor diagnostic criterion.

Anomalies of the ribs and symphysis pubis were less frequently detected (in 3.4% and 1.7% respectively) and are listed under the 'additional observed anomalies in FS'. The reported limb abnormalities (talipes and arthrogryposis) are related to prenatally detected oligo- or anhydramnios, caused by bilateral renal agenesis, in all cases included in this study, and should be regarded as part of the Potter' sequence instead of being characteristic of FS.

5.3.11 Umbilical hernia

Umbilical hernias including omphaloceles are reported in 18.6% of our patients. A low set umbilicus is even more frequently reported (28.8%), so we propose that 'umbilical anomalies' in general should be defined as a minor criterion, comprising both low set umbilical cord and umbilical hernia (including omphalocele). Strict measurements for a low set umbilicus are usually not documented and, to prevent subjective interpretation of this feature, we suggest that it should be documented more precisely (millimetres above the symphisis). The frequency of an abnormal position of the umbilicus could even be higher than observed in the present study since in the presence of an omphalocele the exact position of the umbilicus is difficult to determine. A single

umbilical artery is reported in four patients. It has been suggested that this can be an indicator for renal agenesis (Moore and Persaud, 2003). This was true for three of the four cases (2 bilateral, 1 unilateral renal agenesis). In the fourth patient (33a), there were no renal anomalies detected.

5.3.12 Mental retardation

Only two patients in the present study have a reported delay in development. Of the adult patients, one is attending university. The combination of impaired vision and hearing can lead to a delay in motor development and this instead of an underlying brain defect, should be considered as a causative factor for the reported developmental delay.

There are only a few reports in the literature commenting on the intellectual development of patients affected with Fraser syndrome. Of the 117 cases reviewed by Slavotinek, only 5 were reported to be mentally retarded, whereas a comment on normal development was made in 15 cases (Slavotinek and Tifft, 2002).

Applying mental retardation as a minor diagnostic criterion for FS has led to misdiagnoses in the literature (Butler et al., 1978; Wong et al., 2005), so we propose that mental retardation should no longer been defined as a diagnostic criterion for Fraser syndrome. This will prevent over diagnosis of FS in patients with a combination of mental retardation and congenital malformations that can be part of FS.

There are however structural neurological anomalies reported in our study (encephaloceles), that were already described by Zehender as being a feature of FS (Zehender et al., 1872). The reported frequency of this anomaly is however too low to be defined as a minor diagnostic criterion.

5.3.13 Cardiac abnormalities

Congenital cardiac malformations have been frequently reported as a feature of FS (Aqeel and Al-Alaiyan, 1999; Bieber et al., 1982; Boyd et al., 1988; Brownstein et al., 1976; Burn and Marwood, 1982; Chattopadhyay et al., 1993; Hambire et al., 2003; Lurie and Cherstvoy, 1984; Ramsing et al., 1990). They have however not been defined as a diagnostic criterion before. Heart defects have been documented in six of our cases (Table 3.13). A patent ductus ovale and ductus arteriosis, both observed in two cases,

can be associated with prematurity. This was however not the case in patients 25 and 33 who both died in the neonatal period after a term pregnancy. Apart from the patent ductus ovale and ductus arteriosis, cardiomegaly was also observed at post-mortem investigations. Since heart defects are reported in 10% of the cases in the present study, this feature will be defined as a minor diagnostic criterion.

5.3.14 Gastro-intestinal malformation

Although frequently reported in the literature as a feature of FS, (Bierich et al., 1991; Boyd et al., 1988; Ford et al., 1992; Gattuso et al., 1987; Hing et al., 1990; Koenig and Spranger, 1986; Mina et al., 1988; Ramsing et al., 1990; Woodhead and Hall, 1990), gastro intestinal abnormalities have thus far not been reported as a diagnostic criterion for FS. We found however that anorectal malformations (anal stenosis, anal atresia, imperforate anus and anteriorly placed anus) are frequently reported in the present study (32.2%) and should definitely be defined as a minor diagnostic criterion.

5.3.14.1 Anal abnormalities

Most anorectal malformations result from abnormal development of the urorectal septum, resulting in incomplete separation of the cloaca into urogenital and anorectal portions.

5.3.14.2 Anal agenesis

The anal canal ends blindly or there might be an ectopic opening or a fistula that opens into the perineum. Anal agenesis with a fistula to cervix and vagina has been described in a few of our patients and may be the result of an incomplete separation of the cloaca by the urorectal septum.

5.3.14.3 Anal stenosis

The anus is in the normal position but the anus and anal canal are narrow. This can be caused by a slight dorsal deviation of the urorectal septum as it grows caudally to fuse with the cloacal membrane, resulting in a small anal canal and membrane.

5.3.14.4 Imperforate anus

The anus is in the normal position, but a thin layer of tissue separates the anal canal from the exterior. This is the result of a failure of the anal membrane to perforate at the end of the 8th week of gestation.

5.4 New insights in the pathogenesis of Fraser syndrome

5.4.1 Urogenital defects

Slavotinek and Tifft (2002) suggested that the urogenital abnormalities observed in FS patients might be part of the MURCS "association" (Műllerian agenesis, Renal agenesis/ ectopy, Cervical somite dysplasia) or Bardet- Biedl syndrome. The results of the present study do however not support this idea. MURCS association often involves skeletal malformations that are not observed in our study group (Guerrier et al., 2006). The genital anomalies observed in BBS, do however show some overlap with those observed in FS (Slavotinek and Biesecker, 2000).

5.4.2 Uro-Rectal Septum Malformation (URMS)

The co-occurrence of gastrointestinal abnormalities, genital and renal anomalies in FS show more resemblance of the Urorectal Septum Malformation Sequence (URMS). This disorder was first described by Escobar, who reported on 6 female patients with a specific pattern of abnormalities of the urogenital and lower intestinal tract (Escobar et al., 1987). The anomalies involved ambiguous genitalia, lack of perineal openings, Műllerian duct agenesis and urinary tract anomalies. The most widely accepted theories of the pathogenesis of the URSM suggest deficiencies in the mesoderm migrating into the caudal region. The same idea has been postulated for the observed displacement of the umbilicus, which is also a frequently observed anomaly in FS (Ramsing et al., 1990). Another hypothesis suggests that URSM is the result of an abnormality in the septation of the primitive urorectal septum (Wheeler and Weaver, 2001).

The urinary and genital systems are closely related in their development. The lower urinary system begins as a cloaca. By the sixth week of gestation the urorectal septum fuses with the cloacal membrane, dividing the cloacal cavity into urogenital sinus anteriorly and rectum posteriorly. Apoptosis of the cloacal membrane results in a

urogenital sinus and a rectum with openings to the outside (Sasaki et al., 2004). Failure of the urorectal septum to divide the cloaca or to approach the cloacal membrane leads to the URSM, which subsequently blocks normal development of internal and external genitalia and urinary tract.

5.4.3 BMPs

Although it is too early to present any genotype- phenotype correlations, it is interesting to see from the preliminary data that skull ossification defects, bilateral renal agenesis, abnormal position of umbilicus and anorectal defects are more frequently observed in the FS patients with a *FRAS1* mutation.

FRAS1 is different from the other ECM related proteins in that the N-terminus of FRAS1 contains motifs that are not found in ECM3 or in the other FREM proteins. These chordin-/ kielin-like domains have been associated with modulation of BMP and other TGFβ related protein activity, suggesting that FRAS1 may possibly modulate their activity within the extracellular matrix.

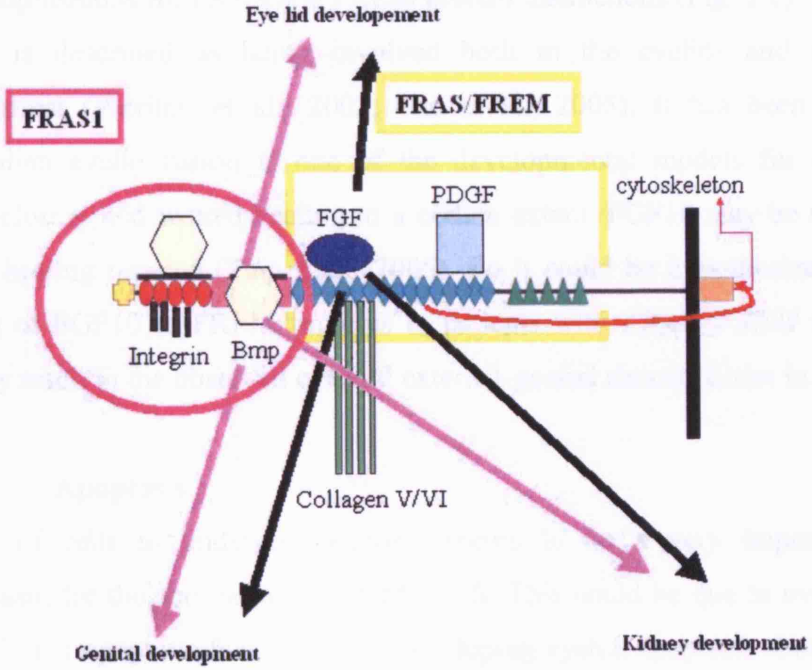

Figure 5.2: Possible domain interactions of FRAS/FREM proteins

BMPs are recognised to have a vital role in metanephrogenesis and in controlling interdigital apoptosis (Dudley et al., 1999; Guha et al., 2002). BMP4 is known to act as

a signal in an epithelial-mesenchymal interaction with Sonic Hedgehog (Shh) in the early phase of hindgut formation. (Sasaki et al., 2004). BMP4 also seems to be involved in the Shh signalling pathway that regulates the patterning and outgrowth of the external genitalia, comparable to that of the development of the limb buds (Perriton et al., 2002). BMP7 plays an important role during the ontogeny of mammalian eye and kidney (Jena et al., 1997). Homozygous mutant animals for BMP7 exhibit renal hypoplasia and eye defects ranging from microphthalmia to anophthalmia (Godin et al., 1998).
Preliminary results from immunohistochemistry experiments suggest that the chordin-like domains do indeed interact with BMPs. (Jadeja, personal communication).

5.4.4 NG2-like domains

The FRAS/FREM proteins have a core CSPG region similar to that found in NG2.
The CSPG domains in NG2 are known to bind bFGF and PDGF-AA with high affinity (Goretzki et al., 1999). They are also capable of diverse interactions with other extra cellular components (Burg et al., 1996; Tillet et al., 2002), many of which may have direct implications for FRAS and FREM protein interactions (Fig. 5.2).
FGF10 is described as being involved both in the eyelid- and external genital development (Perriton et al., 2002; Tao et al., 2005). It has been postulated that mammalian eyelid fusion is one of the developmental models for epidermal hole/ wound closure and wound healing to a certain extent. FGF10 may be required for this wound healing process (Tao et al., 2005). So it could be hypothesized that impaired binding of FGF10 to FREM proteins in patients with *FRAS/ FREM* mutations could possibly result in the observed eye and external-genital abnormalities in FS.

5.4.5 Apoptosis

Failure of cells to undergo apoptosis seems to be a very important underlying mechanism for the anomalies observed in FS. This could be due to over expression of survival/ anti-apoptotic factors. In the developing eyelid, apoptosis should take place to separate the eyelids around week 26. Failure of this process could explain the cryptophthalmos. Atresia of the external ear could be explained in the same way; the meatal plug is not resorbed due to failure of apoptosis in this area. It has already been

hypothesized that the syndactyly might be a result of failure of apoptosis of the interdigital structures, but confirming data are lacking. The role of apoptosis in urorectal separation, urethral opening and rupture of the anal membrane was postulated by Qi et al. and was confirmed by Sasaki et al.(Qi et al., 2000; Sasaki et al., 2004). Further work assessing apoptosis in mouse models is however requested.

Another possible explanation for the anal and vaginal atresia could be the intrauterine bleb formation around these areas. The gastrointestinal-, internal genital and urinary tract anomalies cannot however be explained by this mechanism.

5.5 Conclusions

5.5.1 Clinical analysis

This thesis presents the analysis of the clinical and molecular data of 59 patients with Fraser syndrome. Since our clinical results differ from previous reports in several aspects, we propose modification of diagnostic criteria for Fraser syndrome. There are six major diagnostic criteria (cryptophthalmos spectrum, syndactyly, urinary tract anomalies, ambiguous genitalia, laryngeal /tracheal abnormalities and a positive family history) and six minor criteria (anorectal malformations, dysplastic ears, nasal anomalies, skull ossification defects, umbilical abnormalities and cardiac malformations). The diagnosis of FS can now be made when 3 major; 2 major and 2 minor; or 1 major and 3 minor diagnostic criteria are present in a patient (Table 5.11).

Major Criteria	**Minor criteria**
Syndactyly	Anorectal defects
Cryptophthalmos spectrum	Dysplastic ears
Urinary tract abnormalities	Nasal anomalies
Ambiguous genitalia	Skull ossification defects
Laryngeal and tracheal anomalies	Umbilical abnormalities
Positive family history	Cardiac abnormalities

Table 5.11: Revised diagnostic criteria for Fraser syndrome

5.5.2 Molecular analysis

5.5.2.1 *FRAS1/ FREM2*

Results of linkage studies for candidate genes for Fraser syndrome showed evidence of linkage to *FRAS1* for ten families and to *FREM2* for four families. Three of these families (10, 11, and 14) showed evidence of linkage to both *FREM1* and *FREM2.* Affected patients who are homozygous for different loci may however only be so "by descent" rather than "by disease" given the high degree of consanguinity in these families (Woods, 2005).

Sequencing of *FRAS1* identified mutations in six families. Three families had nonsense mutations, two had missense mutations and in one non-consanguineous family we have thus far identified only a single nonsense mutation. These results are different from the *FRAS1* mutations reported so far, which were all nonsense mutations (McGregor et al., 2003). One of the patients with a *FRAS1* mutation belonged to one of the three families that showed evidence of linkage to both *FRAS1* and *FREM2.*

The common *FREM2* mutation (E1972K) was identified in two further patients from a multiple inbred Spanish Gypsy family after the restriction enzyme test (*BsrGI* digestion of exon 5) showed changes that suggested the common mutation (Fig. 4.1) Subsequent sequencing of exon 5 confirmed the presence of this mutation. Sequencing of *FREM2,* for the four families that were consistent with linkage to *FREM2* revealed thus far no abnormalities. The restriction enzyme test (*BsrGI* digestion of exon5, *FREM2*) was performed for all non-*FREM2* FS patients included in this study, but did not identify any further cases with the E1972K mutation.

	Fam	Origin	Gene	Exon	Mutation	Effect
1	1*	Middle East	*FRAS1*	31	4271C>G	S1424X
2	2*	Lebanon	*FRAS1*	29	3799C>T	Q1267X
3	3*	Middle East	*FRAS1*	41	5575insT	Frame shift
4	4*	Middle East	*FRAS1*	57	8602C>T	Q2868X
5	5	Netherlands	*FRAS1*	74	11544delC	L3848X
6	6*	Australia	*FRAS1*	60	9013C>T	Q3005X
7	11	Turkey	*FRAS1*	63	9627C>A	Y3209X
8	20	Canadian	*FRAS1*	74	11455dupl(49bp)	Frame shift
9	23	United Emirates	*FRAS1*	63	9524A>C	Y3175S
10	34	Poland	*FRAS1*	29	3730C>T	R1244X
11	40	United States	*FRAS1*	29	3877T>C	H1293Y
	40	United States	*FRAS1*	65	10153T>G	Y3385D
12	7	Spanish Gyspies	*FREM2*	5	5914G>A	E1972K
13	8**	Spanish Gyspies	*FREM2*	5	5914G>A	E1972K
14	9**	Spanish Gyspies	*FREM2*	5	5914G>A	E1972K

Table 5.12: *FRAS/FREM* mutations identified in the families included in this study. (*Mutations identified by McGregor, 2002, ** Mutations identified by Jadeja, 2005)

5.5.2.2 *FREM1/ GRIP1*

Two families showed evidence of linkage to *FREM1* for two markers and two families could possibly be linked to *GRIP1*. However, further markers need to be tested before sequencing of these genes will be started.

5.5.2.3 Other candidate genes

Three families included in this study did not show evidence of linkage to any of the candidate genes, suggesting that there could be other genes involved in FS.

5.5.3 Updating previous data

The present study reveals that *FRAS1* comprises 4012 amino acids. The Ensembl sequence that was used for sequence analysis in the present project was aligned with the Swiss protein sequence (used by McGregor, 2003) and resulted in one extra amino acid in exon 24 and four extra amino acids in exon 54. The latter seems to result from a fusion of the previously designated exons 54 and 55. This means a slight alteration of the positions of the *FRAS1* mutations that were identified in families 1, 2, 3, 4, and 6 as reported by McGregor (2003). Revised locations are summarized in Table 5.12 and illustrated in the *FRAS1* sequence in Appendix 1.

5.5.3.1 Intrafamilial variability

Intrafamilial variability has been observed in a few families included in this study (7, 25, 28, 33, and 38). Rousseau already commented on the intrafamilial variability of family 32 (Rousseau et al., 2002). Variability of the phenotype, especially in case of renal abnormalities can have serious prognostic consequences.

5.5.3.2 Life expectancy

The oldest patient included in the present study is 51 years. She is one of the two patients that were originally described by Fraser in 1962. Slavotinek (2002) refers to the oldest Fraser syndrome cases being alive in their fourth decade. These patients were however diagnosed with isolated cryptophthalmos without any other clinical features suggestive of FS (Hancheng, 1986).

5.5.4 Clinical versus molecular analysis

The results of this project illustrate the importance of implementing clinical data in molecular work and vice versa. The results of the clinical study revealed that families with *FRAS1* mutations had more frequently reported skull ossification defects, umbilical-, urinary tract and anorectal malformations than patients without a *FRAS1* mutation.

The molecular results on the other hand provide information about possible effects of the mutations. Disturbance of FRAS1-BMP4 interactions seems to be an interesting

hypothesis for the observed clinical features in patients with a *FRAS1* mutation. Although genotyping results show evidence of linkage to *FREM1* and *GRIP1* in a few families, sequencing for human mutations in *FREM1* and *GRIP1* has not been performed yet. Further markers will be tested before this will be started.

5.6 Future directions

5.6.1 Genotyping

New consanguineous families need to be genotyped for the candidate genes before further sequencing will be started

5.6.2 Sequencing

New incoming samples of non-consanguineous families could be screened for *FRAS1* mutations first when a patient is reported to have the 'typical *FRAS1* features'.

The results of this study indicate various hotspots in *FRAS1* (exons 29, 63). Focussing on these exons first could prevent the time-consuming screening of all 74 exons of *FRAS1*.

FREM2 sequencing has not been completed yet and there may well be other mutations detected in the future apart from the common E1972K mutation that has been identified in 3 families included in this project. It is remarkable that the *FREM2* mutations were only identified in Spanish Gypsy families, suggesting a founder mutation. However, patients 8 and 9 were homozygous with respect to different alleles for an intronic SNP. Therefore, it is likely that the mutation arose independently in these two families and does not represent an ancestral mutation (Jadeja et al., 2005).

5.6.3 *BsrGI* enzyme test

Although a founder effect is less likely to cause the *FREM2* mutations in the Spanish gypsy families, new incoming samples from Spanish Gypsies will still be checked for

this common E1972K mutation in *FREM2* with the *BsrGI* enzyme test before genotyping or sequencing will be performed.

5.6.4 Immunohistochemistry

It will be interesting to explore the 'Fras1- Bmp4 interaction' hypothesis further by carrying out immunohistochemistry experiments to look for the co-expression of Fras1 and BMP4 in the kidneys and urogenital area.

5.6.5 Digenic inheritance

Thus far we only identified a single nonsense mutation in the two affected children of the non-consanguineous family 34. The mother carries the same mutation. Since *FRAS1* mutation screening did not reveal any further abnormalities, it could be hypothesized that the second mutation will be identified in another gene, illustrating the principle of digenic inheritance that has been described in several human diseases (Ming and Muenke, 2002). In digenic disorders, a single individual has mutations in two unlinked genes. The disease phenotype is caused by the combination of these two genetic hits and is not apparent in individuals who carry only one of these gene alterations.

The case reported by Roizenblatt et al. (1982) could also be explained by this principle. Althought not fulfilling the revised diagnostic criteria, the affected patient shows some of the features of FS (agenesis of the right kidney, ocular hypertelorism, primary telecanthus, cleft nose with absent tip, broad nasal root, complete absence of the left upper lid, high arched palate, and sensorineural deafness). The mother of this patient is reported to have minor facial anomalies. The more severe phenotype in the affected child could possibly be the result of multiple genetic alterations, whereas the presence of only one abnormal gene (as could be suggested for the mother), may give rise to either a mild manifestation or no clinical abnormality.

5.6.6 Further candidate genes

To date, there are two cytogenetic abnormalities described in patients with FS. The patient described by Schauer et al. (1990) has a 46,XY,inv(9)(p11q21). It might be

interesting to repeat the chromosomal analysis to see if modern techniques could detect a more precisely defined breakpoint since *FREM1* is located in this area.

The second case, described under family 1 in this thesis, had a balanced translocation; 46,XX t(2;16)(p15;q22). The healthy mother had the same karyotype. Although a disease causing *FRAS1* mutation has been identified in this patient, it would still be interesting to see if the translocation breakpoints harbour any modifier genes that could explain the intrafamilial variability that is frequently observed in this condition.

5.6.7 Diagnostic service

Offering a diagnostic molecular service for Fraser Syndrome would facilitate further research by gaining more information about the clinical features observed in FS. Reliable criteria for phenotype assessment on the other hand, are essential to direct mutation analysis in FS for non-consanguineous cases. This will also prevent the time-consuming mutation analysis of *FRAS1* (74 exons) in cases where phenotypic assessment indicates a possible molecular defect in one of the smaller candidate genes.

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Appendix 1


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Ex 2        R
3    91 CAGGATTCCTTGTTGGCGGATGCCACAATTTGGAAGCCCGATTCATGCCAGAGCTGCCGT
     31 -Q-=D=-S--L--L--A--D--A--T--I--W--K--P--D--S--C--Q--S--C--R-
Ex 3              S        VWC domains
    151 TGCCATGGTGATATTGTTATCTGCAAACCTGCTGTTTGCAGAAACCCTCAATGTGCCTTT
     51 -C--H--G-=D=-I--V--I--C--K--P--A--V--C--R--N--P--Q--C--A--F-

    211 GAGAAGGGAGAAGTGCTTCAAATAGCTGCCAACCAATGCTGTCCTGAGTGTGTTTTGAGG
     71 -E--K--G--E--V--L--Q--I--A--A--N--Q--C--C--P--E--C--V--L--R-
Ex 4
    271 ACTCCAGGATCTTGCCATCATGAAAAGAAAATCCATGAGCATGGGACAGAATGGGCCTCT
     91 -T--P--G--S--C--H--H--E--K--K--I--H--E--H--G--T--E--W--A--S-

    331 TCTCCATGTAGTGTGTGCTCTTGCAATCATGGGGAAGTCCGATGTACCCCCCAACCATGC
    111 -S--P--C--S--V--C--S--C--N--H--G--E--V--R--C--T--P--Q--P--C-
Ex 5
    391 CCACCGCTGTCATGTGGACACCAGGAGCTGGCATTCATCCCTGAAGGAAGCTGCTGCCCA
    131 -P--P--L--S--C--G--H--Q--E--L--A--F--I--P--E--G--S--C--C--P-

    451 GTTTGTGTGGGCCTTGGGAAACCCTGTTCCTATGAAGGCCATGTGTTTCAGGATGGGGAG
    151 -V--C--V--G--L--G--K--P--C--S--Y--E--G--H--V--F--Q--D--G--E-
Ex 6
    511 GACTGGCGGCTGAGCCGGTGTGCCAAATGTCTGTGTAGAAATGGGGTTGCCCAGTGCTTC
    171 -D--W--R--L--S--R--C--A--K--C--L--C--R--N--G--V--A--Q--C--F-
                                                                Y
    571 ACAGCTCAGTGTCAGCCTCTATTTTGTAACCAGGATGAGACTGTAGTCCGAGTCCCTGGA
    191 -T--A--Q--C--Q--P--L--F--C--N--Q--D--E--T--V--V--R--V-=P=--G-
Ex 7                                                          Y
    631 AAATGTTGCCCGCAGTGCTCTGCAAGATCCTGCTCTGCAGCTGGCCAAGTATACGAGCAT
    211 -K--C--C--P--Q--C--S--A--R--S--C--S--A--A--G--Q--V-=Y=-E--H-
          K                                    R
    691 GGTGAGCAGTGGAGCGAAAATGCCTGCACCACGTGTATATGTGACCGGGGTGAGGTCAGG
    231 -G--E--Q--W--S--E--N--A--C--T--T--C-=I=-C--D--R--G--E--V--R-
Ex 8
    751 TGTCACAAGCAGGCCTGCCTGCCCCTGAGATGCGGAAAGGGTCAGAGCAGGGCTCGGCGT
    251 -C--H--K--Q--A--C--L--P--L--R--C--G--K--G--Q--S--R--A--R--R-
Ex 9
    811 CATGGGCAATGCTGTGAGGAATGTGTGTCTCCTGCCGGGAGCTGCTCCTATGATGGAGTT
    271 -H--G--Q--C--C--E--E--C--V--S--P--A--G--S--C--S--Y--D--G--V-

    871 GTGCGGTACCAGGACGAAATGTGGAAGGGCTCGGCCTGTGAGTTCTGCATGTGTGATCAT
    291 -V--R--Y--Q--D--E--M--W--K--G--S--A--C--E--F--C--M--C--D--H-

    931 GGCCAAGTGACCTGCCAGACTGGAGAGTGTGCCAAAGTGGAGTGTGCCCGGGATGAAGAA
    313 -G--Q--V--T--C--Q--T--G--E--C--A--K--V--E--C--A--R--D--E--E-
Ex 10
    991 TTAATTCACTTAGATGGAAAGTGTTGTCCTGAATGCATTTCAAGGAATGGTTATTGTGTT
    331 -L--I--H--L--D--G--K--C--C--P--E--C--I--S--R--N--G--Y--C--V-
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  1051 TATGAAGAAACTGGAGAATTTATGTCATCAAATGCTAGTGAAGTTAAACGTATTCCAGAG
   351 -Y--E--E--T--G--E--F--M--S--S--N--A--S--E--V--K--R--I--P--E-
Ex 11
  1111 GGAGAGAAGTGGGAAGATGGCCCTTGCAAGGTGTGTGAGTGCCGAGGGGCTCAGGTAACT
   371 -G--E--K--W--E--D--G--P--C--K--V--C--E--C--R--G--A--Q--V--T-
Ex 12
  1171 TGCTACGAGCCCTCTTGCCCACCATGTCCAGTGGGCACACTGGCCTTAGAGGTGAAGGGA
   391 -C--Y--E--P--S--C--P--P--C--P--V--G--T--L--A--L--E--V--K--G-
                                                                M
  1231 CAGTGCTGTCCAGACTGCACATCAGTTCATTGCCATCCAGATTGTTTGACATGCTCTCAG
   411 -Q--C--C--P--D--C--T--S--V--H--C--H--P--D--C--L--T--C-=S=--Q-
Ex 13
  1291 TCTCCAGACCACTGTGACCTCTGCCAAGATCCTACCAAGTTACTGCAGAATGGATGGTGT
   431 -S--P--D--H--C--D--L--C--Q--D--P--T--K--L--L--Q--N--G--W--C-
                                                      W
  1351 GTGCACAGCTGTGGACTGGGTTTTTACCAAGCTGGCAGTCTCTGTCTAGCCTGCCAGCCC
   451 -V--H--S--C--G--L--G--F--Y--Q--A--G--S--L--C-=L=-A--C--Q--P-
Ex 14
  1411 CAGTGCTCCACGTGTACCAGTGGGCTGGAGTGCTCATCCTGCCAGCCTCCCCTGCTGATG
   471 -Q--C--S--T--C--T--S--G--L--E--C--S--S--C--Q--P--P--L--L--M-

  1471 CGGCACGGGCAGTGTGTGCCTACCTGTGGGGACGGCTTCTACCAAGATCGCCATTCCTGT
   491 -R--H--G--Q--C--V--P--T--C--G--D--G--F--Y--Q--D--R--H--S--C-

  1531 GCAGTCTGCCATGAGTCCTGTGCAGGTTGCTGGGGCCCAACGGAGAAGCACTGCTTGGCC
   511 -A--V--C--H--E--S--C--A--G--C--W--G--P--T--E--K--H--C--L--A-
Ex 15                                R
  1591 TGCAGAGATCCCCTCCACGTGCTGAGCGATGGCGGCTGTGAGAGCAGCTGTGGAAAAGGC
   531 -C--R--D--P--L--H--V--L--R--D--G--G--C--E--S--S--C--G--K--G-
                                                                Y
  1651 TTCTACAACAGGCAGGGCACCTGTAGCGCTTGTGACCAATCCTGTGACAGTTGTGGCCCT
   551 -F--Y--N--R--Q--G--T--C--S--A--C--D--Q--S--C--D--S--C--G--P-
Ex 16
  1711 AGTAGCCCCAGGTGTCTTACCTGTACTGAGAAGACAGTGCTGCATGATGGGAAATGCATG
   571 -S--S--P--R--C--L--T--C--T--E--K--T--V--L--H--D--G--K--C--M-

  1771 TCTGAATGCCCTGGCGGGTACTATGCTGATGCCACTGGCAGGTGCAAAGTTTGTCATAAC
   591 -S--E--C--P--G--G--Y--Y--A--D--A--T--G--R--C--K--V--C--H--N-
Ex 17
  1831 TCATGTGCCAGCTGCTCTGGGCCCACACCCTCTCACTGTACAGCCTGCAGCCCCCCCAAG
   611 -S--C--A--S--C--S--G--P--T--P--S--H--C--T--A--C--S--P--P--K-
                                                                Y
  1891 GCTCTGCGTCAAGGCCACTGTCTGCCCCGCTGTGGAGAGGGTTTCTACTCTGACCATGGA
   631 -A--L--R--Q--G--H--C--L--P--R--C--G--E--G--F--Y--S--D--H--G-

  1951 GTCTGCAAAGCCTGTCACTCCTCCTGCCTGGCTTGTATGGGTCCCGCACCCTCTCACTGT
   651 -V--C--K--A--C--H--S--S--C--L--A--C--M--G--P--A--P--S--H--C-
Ex 18                                                    R
  2011 ACTGGGTGTAAGAAGCCAGAGGAAGGACTGCAAGTGGAGCAGCTGTCTGACGTGGGCATC
   671 -T--G--C--K--K--P--E--E--G--L--Q--V--E--Q--L--S-=D=-V--G--I-
                                                                M
  2071 CCCTCTGGCGAGTGTCTAGCCCAGTGTAGAGCCCATTTTTACTTGGAGAGCACTGGCATA
   691 -P--S--G--E--C--L--A--Q--C--R--A--H--F--Y--L--E--S--T--G-=I=

  2131 TGTGAAGCTTGCCACCAGTCCTGTTTCAGATGTGCAGGGAAAAGCCCACATAACTGCACA
   711 -C--E--A--C--H--Q--S--C--F--R--C--A--G--K--S--P--H--N--C--T-
Ex 19
  2191 GACTGTGGGCCTTCCCATGTGCTGTTGGATGGGCAGTGCCTCTCCCAGTGCCCAGATGGC
   731 -D--C--G--P--S--H--V--L--L--D--G--Q--C--L--S--Q--C--P--D--G-
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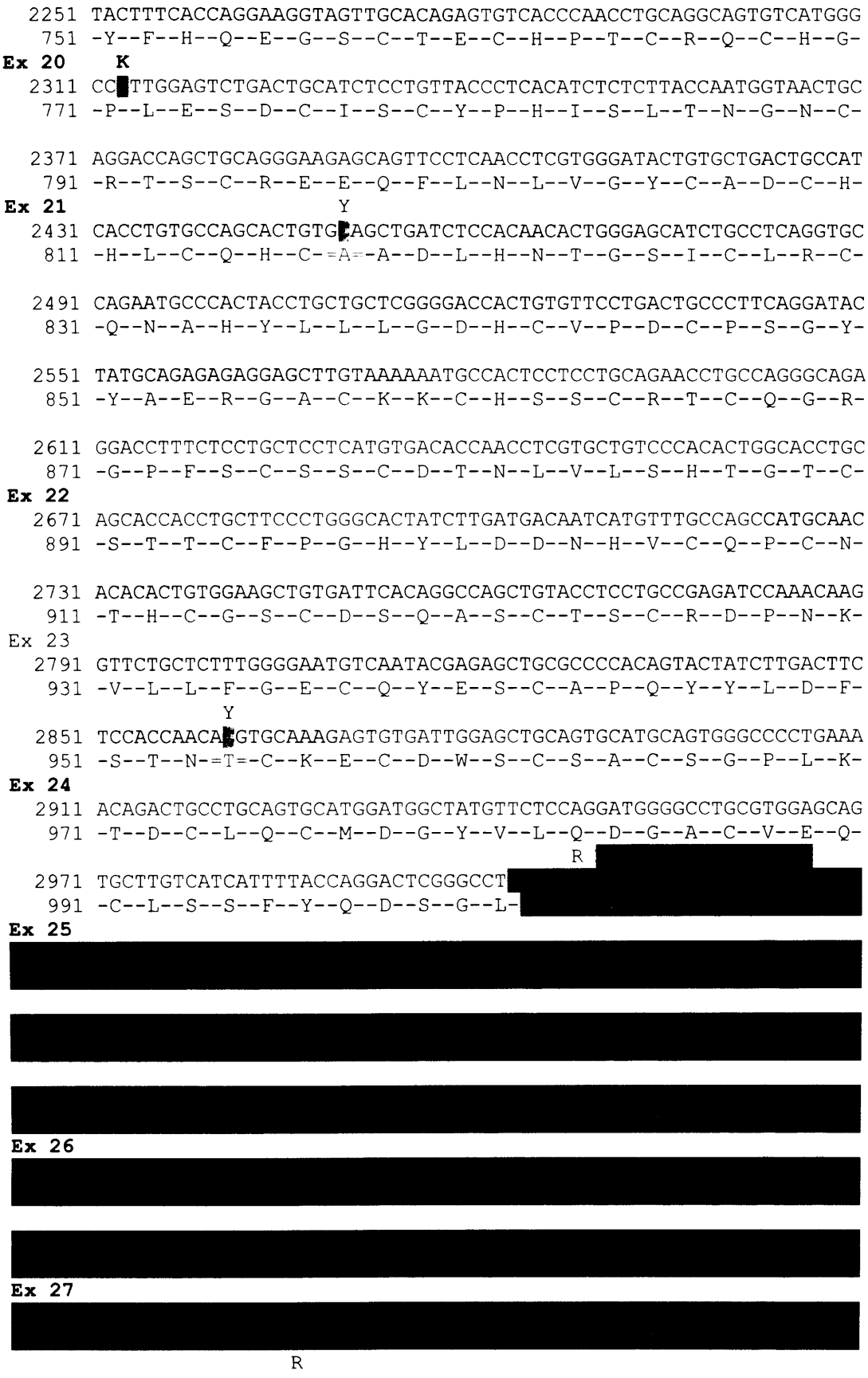
2251 TACTTTCACCAGGAAGGTAGTTGCACAGAGTGTCACCCAACCTGCAGGCAGTGTCATGGG
751 -Y--F--H--Q--E--G--S--C--T--E--C--H--P--T--C--R--Q--C--H--G-
Ex 20
K
2311 CC[redacted]TTGGAGTCTGACTGCATCTCCTGTTACCCTCACATCTCTCTTACCAATGGTAACTGC
771 -P--L--E--S--D--C--I--S--C--Y--P--H--I--S--L--T--N--G--N--C-
2371 AGGACCAGCTGCAGGGAAGAGCAGTTCCTCAACCTCGTGGGATACTGTGCTGACTGCCAT
791 -R--T--S--C--R--E--E--Q--F--L--N--L--V--G--Y--C--A--D--C--H-
Ex 21
Y
2431 CACCTGTGCCAGCACTGTG[redacted]AGCTGATCTCCACAACACTGGGAGCATCTGCCTCAGGTGC
811 -H--L--C--Q--H--C-=A=-A--D--L--H--N--T--G--S--I--C--L--R--C-
2491 CAGAATGCCCACTACCTGCTGCTCGGGGACCACTGTGTTCCTGACTGCCCTTCAGGATAC
831 -Q--N--A--H--Y--L--L--L--G--D--H--C--V--P--D--C--P--S--G--Y-
2551 TATGCAGAGAGAGGAGCTTGTAAAAAATGCCACTCCTCCTGCAGAACCTGCCAGGGCAGA
851 -Y--A--E--R--G--A--C--K--K--C--H--S--S--C--R--T--C--Q--G--R-
2611 GGACCTTTCTCCTGCTCCTCATGTGACACCAACCTCGTGCTGTCCCACACTGGCACCTGC
871 -G--P--F--S--C--S--S--C--D--T--N--L--V--L--S--H--T--G--T--C-
Ex 22
2671 AGCACCACCTGCTTCCCTGGGCACTATCTTGATGACAATCATGTTTGCCAGCCATGCAAC
891 -S--T--T--C--F--P--G--H--Y--L--D--D--N--H--V--C--Q--P--C--N-
2731 ACACACTGTGGAAGCTGTGATTCACAGGCCAGCTGTACCTCCTGCCGAGATCCAAACAAG
911 -T--H--C--G--S--C--D--S--Q--A--S--C--T--S--C--R--D--P--N--K-
Ex 23
2791 GTTCTGCTCTTTGGGGAATGTCAATACGAGAGCTGCGCCCCACAGTACTATCTTGACTTC
931 -V--L--L--F--G--E--C--Q--Y--E--S--C--A--P--Q--Y--Y--L--D--F-
Y
2851 TCCACCAACA[redacted]GTGCAAAGAGTGTGATTGGAGCTGCAGTGCATGCAGTGGGCCCCTGAAA
951 -S--T--N-=T=-C--K--E--C--D--W--S--C--S--A--C--S--G--P--L--K-
Ex 24
2911 ACAGACTGCCTGCAGTGCATGGATGGCTATGTTCTCCAGGATGGGGCCTGCGTGGAGCAG
971 -T--D--C--L--Q--C--M--D--G--Y--V--L--Q--D--G--A--C--V--E--Q-
R
2971 TGCTTGTCATCATTTTACCAGGACTCGGGCCT[redacted]
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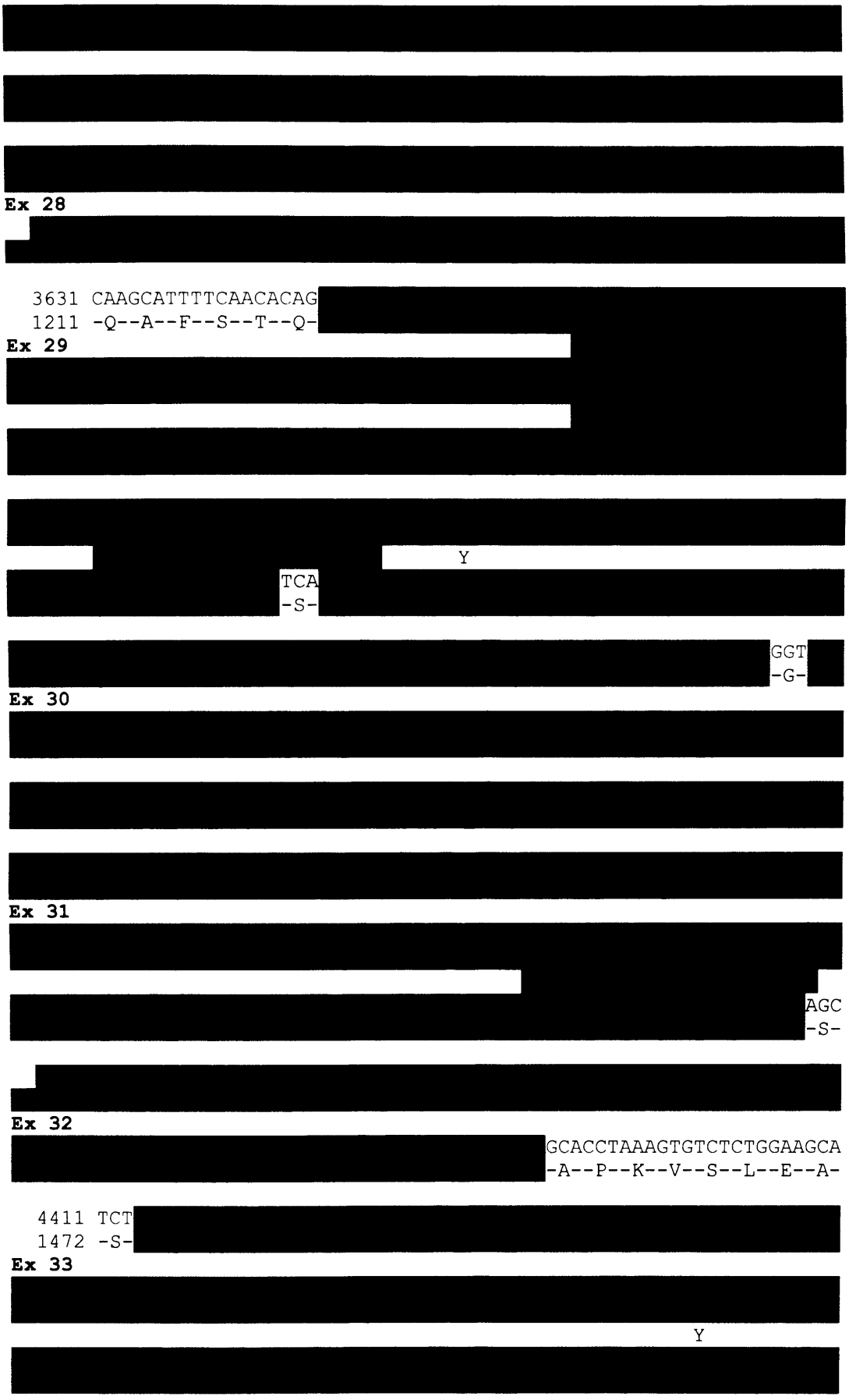
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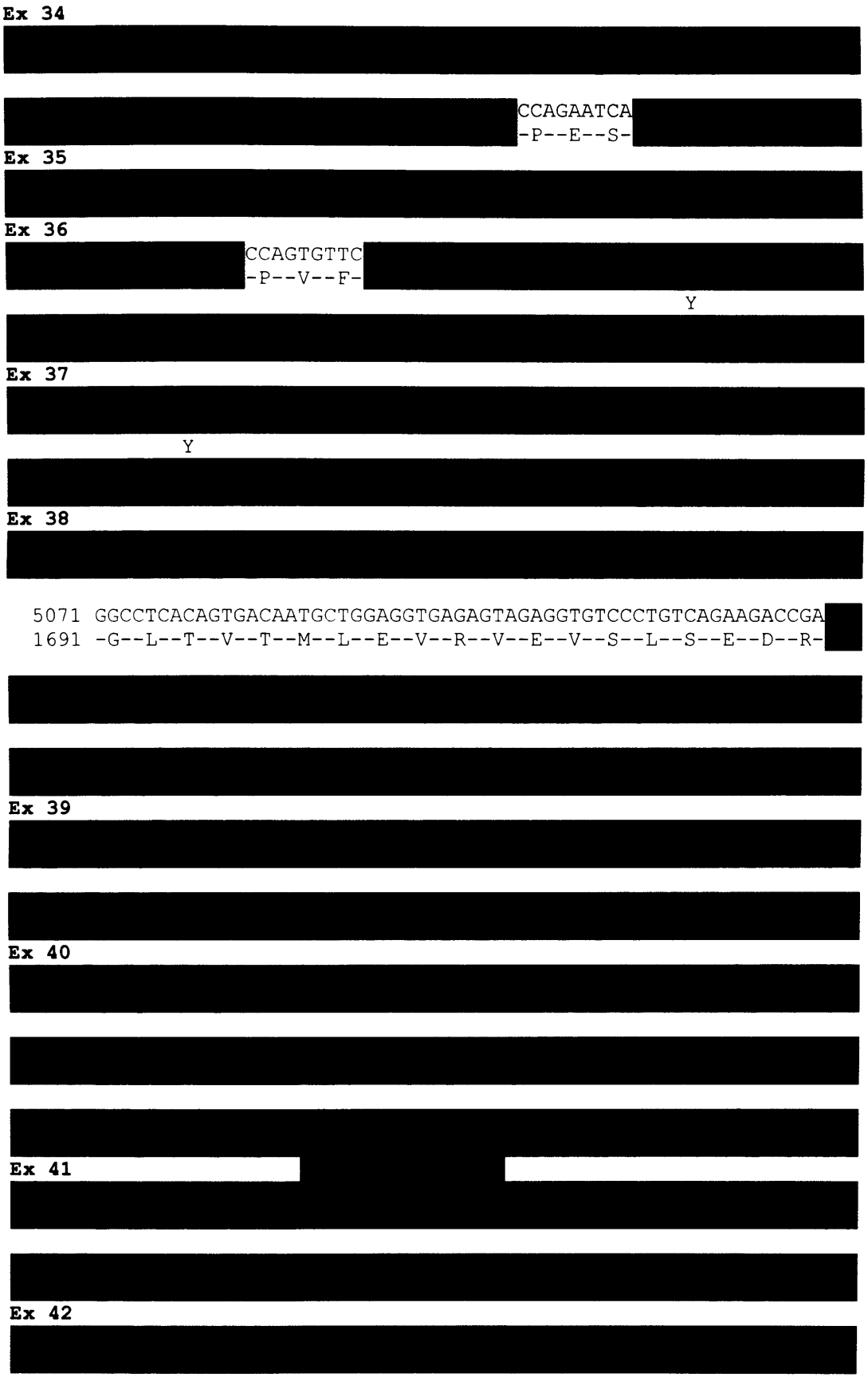
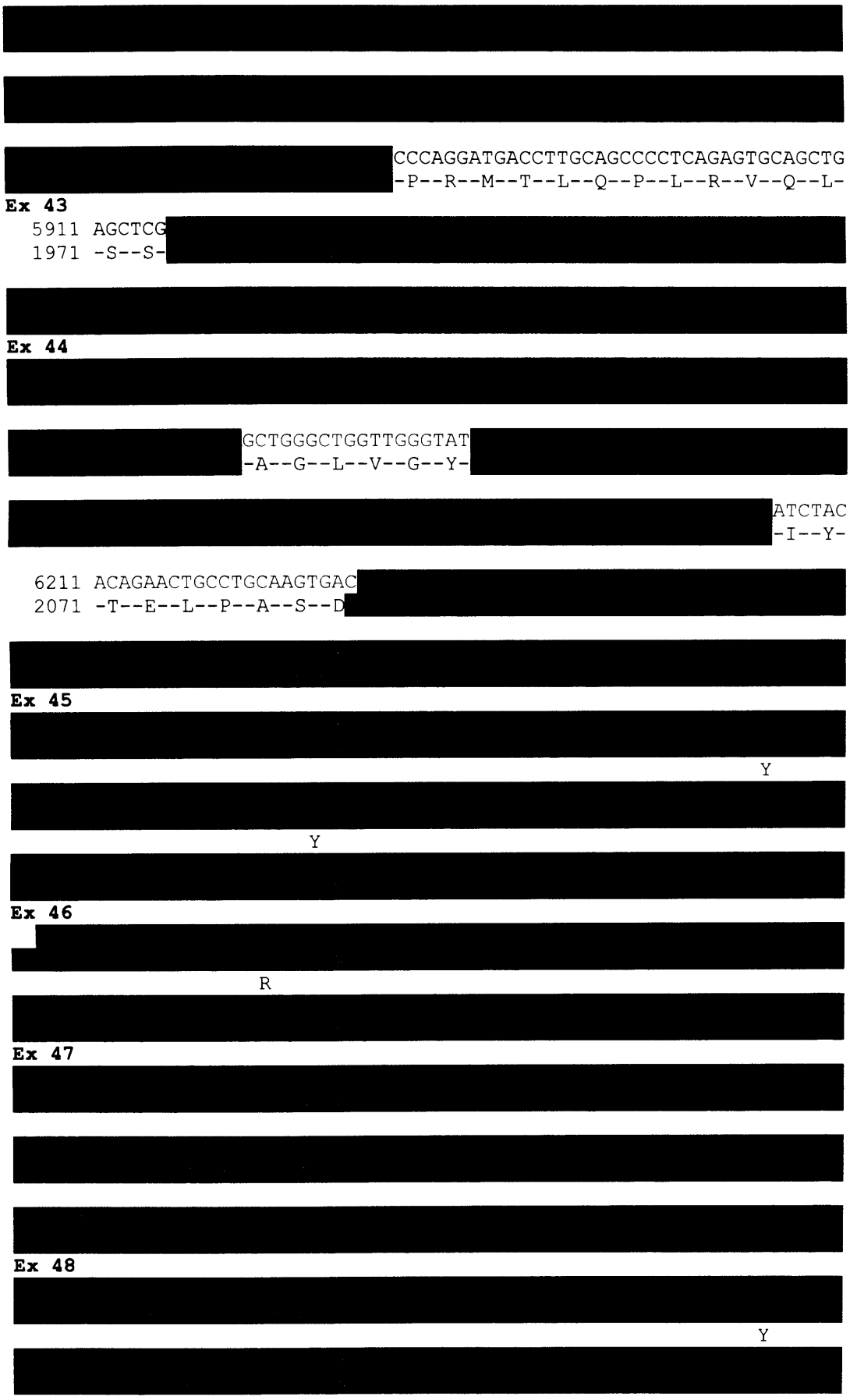
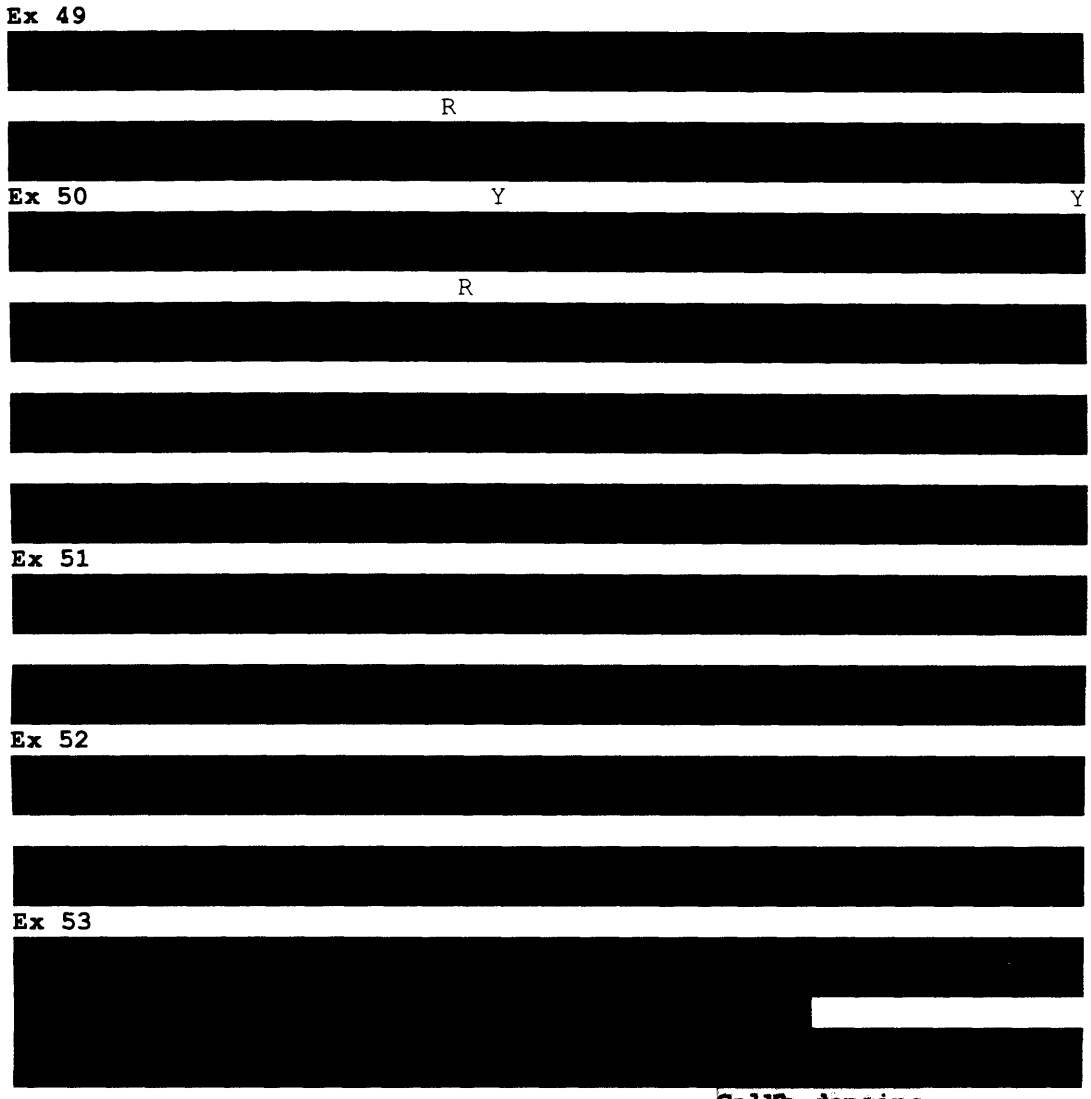
3631 CAAGCATTTTCAACACAG
1211 -Q--A--F--S--T--Q-
Ex 29
Y
TCA
-S-
GGT
-G-
Ex 30
Ex 31
AGC
-S-
Ex 32
GCACCTAAAGTGTCTCTGGAAGCA
-A--P--K--V--S--L--E--A-
4411 TCT
1472 -S-
Ex 33
Y


Ex 34
CCAGAATCA
-P--E--S-
Ex 35
Ex 36
CCAGTGTTC
-P--V--F-
Y
Ex 37
Y
Ex 38
5071 GGCCTCACAGTGACAATGCTGGAGGTGAGAGTAGAGGTGTCCCTGTCAGAAGACCGA
1691 -G--L--T--V--T--M--L--E--V--R--V--E--V--S--L--S--E--D--R-
Ex 39
Ex 40
Ex 41
Ex 42


CCCAGGATGACCTTGCAGCCCCTCAGAGTGCAGCTG
-P--R--M--T--L--Q--P--L--R--V--Q--L-
Ex 43
5911 AGCTCG
1971 -S--S-
Ex 44
GCTGGGCTGGTTGGGTAT
-A--G--L--V--G--Y-
ATCTAC
-I--Y-
6211 ACAGAACTGCCTGCAAGTGAC
2071 -T--E--L--P--A--S--D
Ex 45
Y
Y
Ex 46
R
Ex 47
Ex 48
Y



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 7651 CAGTGGTCACTCATCAGCTTTAAATATACCAGCTACAATGTCAGTGAGAAGGCAGGGTCT
 2551 -Q--W--S--L--I--S--F--K--Y--T--S--Y--N--V--S--E--K--A--G--S-
Ex 54
 7711 GTCAGTGTCACGGTGCAGAGGACTGGGAACCTGAACCAATATGCCATCGTCCTGTGTCGC
 2571 -V--S--V--T--V--Q--R--T--G--N--L--N--Q--Y--A--I--V--L--C--R-

 7771 ACCGAGCAAGGCACCGCCAGCTCCAGCTCCAGGGTCAGCTCCCAACCTGGGCAACAGGAC
 2591 -T--E--Q--G--T--A--S--S--S--S--R--V--S--S--Q--P--G--Q--Q--D-

 7831 TATGTAGAGTATGCTGGCCAGGTCCAGTTTGATGAGCGAGAGGACACCAAGTCCTGCACC
 2611 -Y--V--E--Y--A--G--Q--V--Q--F--D--E--R--E--D--T--K--S--C--T-
Ex 55
 7891 ATTGTCATCAACGATGATGACGTGTTTGAAAATGTTGAGAGTTTCACTGTGGAGCTCAGC
 2631 -I--V--I--N--D--D--D--V--F--E--N--V--E--S--F--T--V--E--L--S-

 7951 ATGCCAGCTTATGCCCTGTTAGGGGAATTCACCCAGGCGAAGGTCATTATCAACGATACC
 2651 -M--P--A--Y--A--L--L--G--E--F--T--Q--A--K--V--I--I--N--D--T-

 8011 GAGGATGAACCCACATTAGAGTTTGACAAGAAGATCTACTGGGTTAACGAGAGCGCTGGT
 2671 -E--D--E--P--T--L--E--F--D--K--K--I--Y--W--V--N--E--S--A--G-
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 8071 TTTCTGTTTGCACCTATTGAAAGAAAAGGAGATGCAAGCAGCATTGTATCTGCAATTTGC
 2691 -F--L--F--A--P--I--E--R--K--G--D--A--S--S--I--V--S--A--I--C-
Ex 56
 8131 TACACAGTCCCTAAGTCAGCTATGGGAAGTAGCCTCTATGCTCTAGAATCAGGCTCTGAT
 2711 -Y--T--V--P--K--S--A--M--G--S--S--L--Y--A--L--E--S--G--S--D-

 8191 TTTAAATCTAGAGGGATGTCTGCCGCGAGTCGTGTGATATTCGGGCCTGGTGTGACCATG
 2731 -F--K--S--R--G--M--S--A--A--S--R--V--I--F--G--P--G--V--T--M-

 8251 TCCACCTGTGATGTCATGCTTATTGATGACAGCGAGTATGAAGAGGAAGAAGAGTTTGAG
 2751 -S--T--C--D--V--M--L--I--D--D--S--E--Y--E--E--E--E--E--F--E-

 8311 ATTGCCTTGGCAGATGCCTCTGACAATGCCCGCATTGGAAGGGTGGCGACAGCCAAGGTG
 2771 -I--A--L--A--D--A--S--D--N--A--R--I--G--R--V--A--T--A--K--V-

 8371 CTCATTAGTGGTCCCAACGATGCCTCGACTGTGTCCCTGGGCAACACGGCTTTCACTGTC
 2791 -L--I--S--G--P--N--D--A--S--T--V--S--L--G--N--T--A--F--T--V-
                Y
 8431 AGTGAGGACGCAGGCACAGTAAAGATTCCAGTTATCCGCCATGGTACTGACCTCTCTACT
 2811 -S--E--D--A--G--T--V--K--I--P--V--I--R--H--G--T--D--L--S--T-
Ex 57
 8491 TTCGCATCTGTCTGGTGTGCAACGCGGCCCTCAGACCCAGCTTCTGCCACACCAGGAGTT
 2831 -F--A--S--V--W--C--A--T--R--P--S--D--P--A--S--A--T--P--G--V-

 8551 GACTACGTTCCCAGCTCTCGGAAGGTGGAATTTGGGCCTGGTGTCATTGAA[redacted]AGTATTGC
 2851 -D--Y--V--P--S--S--R--K--V--E--F--G--P--G--V--I--E--[redacted]--Y--C-
Ex 58
 8611 ACCTTGACTATCTTGGATGACACTCAGTATCCGGTAATTGAAGGACTGGAGACATTTGTG
 2871 -T--L--T--I--L--D--D--T--Q--Y--P--V--I--E--G--L--E--T--F--V-

 8671 GTTTTCCTCAGCTCAGCACAAGGAGCCGAACTGACCAAACCCTTCCAGGCAGTCATTGCA
 2891 -V--F--L--S--S--A--Q--G--A--E--L--T--K--P--F--Q--A--V--I--A-

 8731 ATTAATGACACATTCCAAGATGTGCCCAGCATGCAGTTTGCCAAGGATTTGCTCCTAGTG
 2911 -I--N--D--T--F--Q--D--V--P--S--M--Q--F--A--K--D--L--L--L--V-
Ex 59
 8791 AAGGAGAAGGAGGGTGTCCTGCATGTCCCTATCACTCGGAGCGGAGACCTGAGCTATGAG
 2931 -K--E--K--E--G--V--L--H--V--P--I--T--R--S--G--D--L--S--Y--E-

 8851 TCATCAGTGAGGTGCTATACTCAGAGCCATTCCGCTCAGGTCATGGAGGACTTTGAGGAG
 2951 -S--S--V--R--C--Y--T--Q--S--H--S--A--Q--V--M--E--D--F--E--E-

 8911 AGACAAAATGCAGACTCTTCACGGATTACATTTCTCAAAGGGGACAAAGTGAAGAACTGT
 2971 -R--Q--N--A--D--S--S--R--I--T--F--L--K--G--D--K--V--K--N--C-
Ex 60
 8971 ACGGTCTATATCCACGATGACTCCATGTTTGAGCCAGAGGAA[redacted]AGTTCAGGGTCTACCTC
 2991 -T--V--Y--I--H--D--D--S--M--F--E--P--E--E--[redacted]--F--R--V--Y--L-

 9031 GGCCTTCCTCTTGGAAACCACTGGAGTGGAGCTAGAATTGGAAAGAATAACATGGCCACC
 3011 -G--L--P--L--G--N--H--W--S--G--A--R--I--G--K--N--N--M--A--T-

 9091 ATCACCATATCCAATGATGAAGATGCCCCCACCATTGAGTTTGAAGAAGCTGCATACCAA
 3031 -I--T--I--S--N--D--E--D--A--P--T--I--E--F--E--E--A--A--Y--Q-
Ex 61
 9151 GTCCGGGAACCCGCAGGCCCAGATGCCATTGCGATTCTGAACATCAAGGTGATCCGCAGA
 3051 -V--R--E--P--A--G--P--D--A--I--A--I--L--N--I--K--V--I--R--R-
                                                  K
 9211 GGGGATCAGAACAGGACCTCCAAGGTTCGCTGCAGCACGCGGGATGGCTCTGCCCAGTCT
 3071 -G--D--Q--N--R--T--S--K--V--R--C--S--T--R--D--G--S--A--Q--S-
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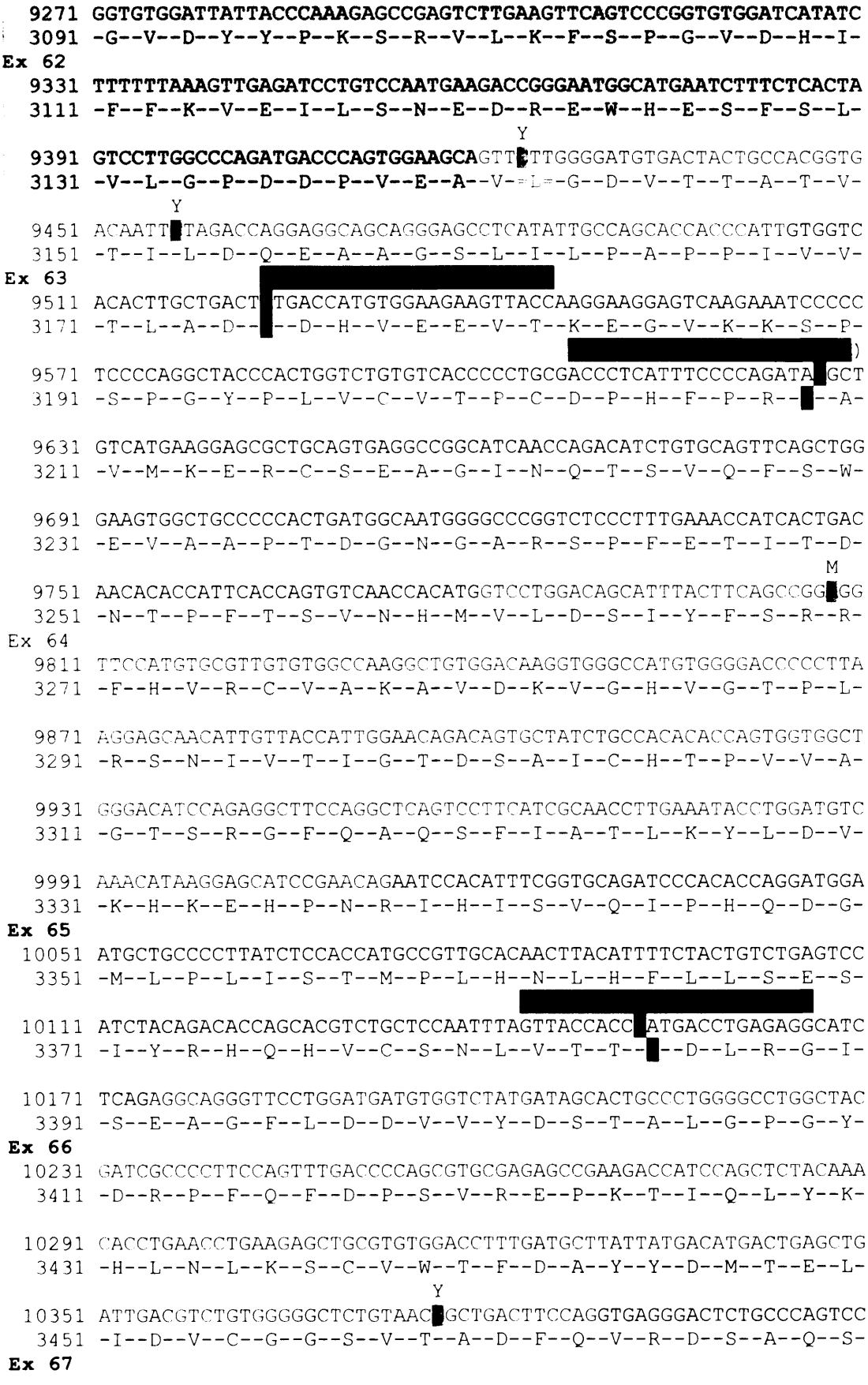
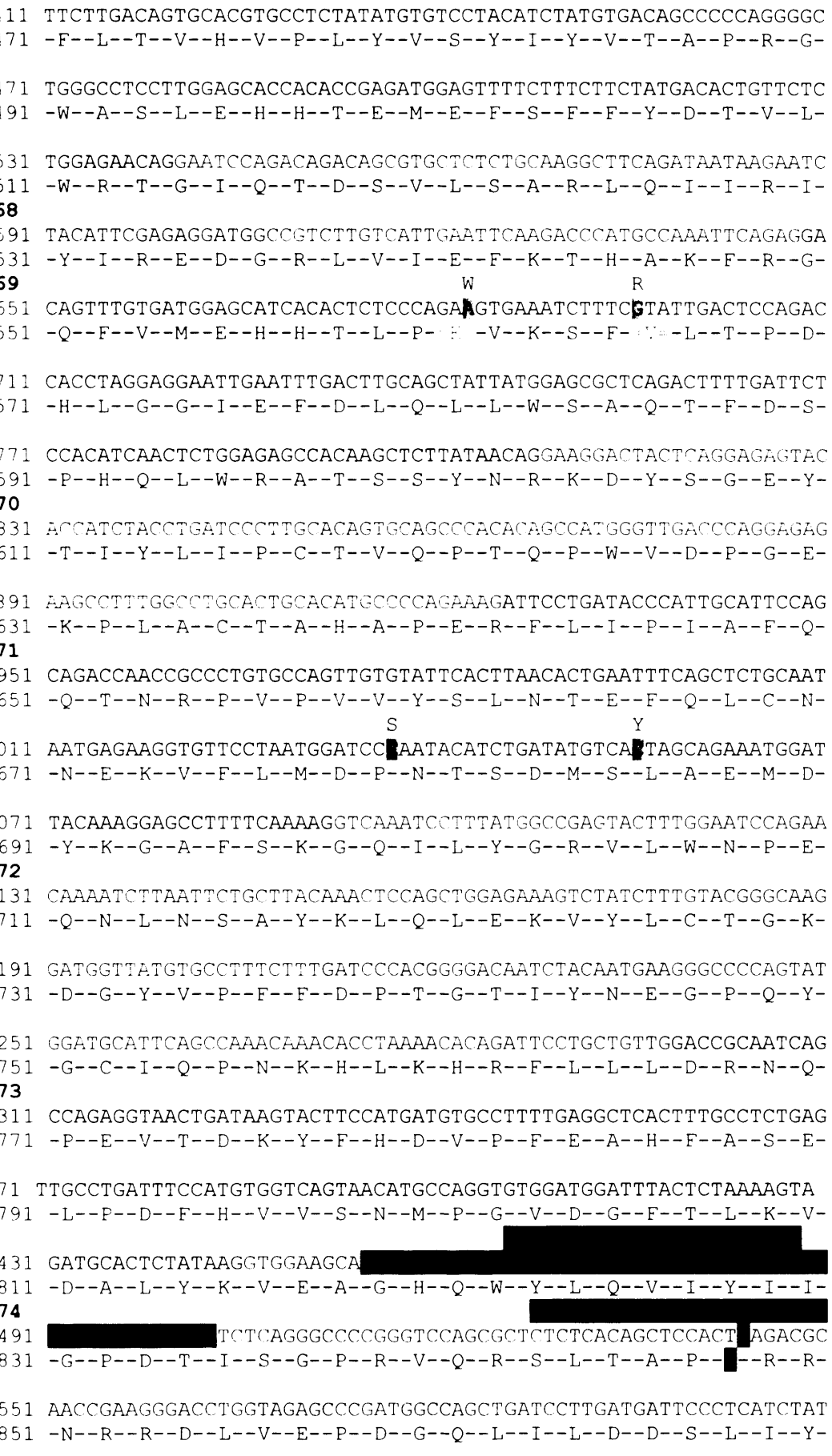

9271 GGTGTGGATTATTACCCAAAGAGCCGAGTCTTGAAGTTCAGTCCCGGTGTGGATCATATC
3091 -G--V--D--Y--Y--P--K--S--R--V--L--K--F--S--P--G--V--D--H--I-
Ex 62
9331 TTTTTTAAAGTTGAGATCCTGTCCAATGAAGACCGGGAATGGCATGAATCTTTCTCACTA
3111 -F--F--K--V--E--I--L--S--N--E--D--R--E--W--H--E--S--F--S--L-
9391 GTCCTTGGCCCAGATGACCCAGTGGAAGCAGTTGTTGGGGATGTGACTACTGCCACGGTG
3131 -V--L--G--P--D--D--P--V--E--A--V--L--G--D--V--T--T--A--T--V-
9451 ACAATTGTAGACCAGGAGGCAGCAGGGAGCCTCATATTGCCAGCACCACCCATTGTGGTC
3151 -T--I--L--D--Q--E--A--A--G--S--L--I--L--P--A--P--P--I--V--V-
Ex 63
9511 ACACTTGCTGACTGTGACCATGTGGAAGAAGTTACCAAGGAAGGAGTCAAGAAATCCCCC
3171 -T--L--A--D--D--H--V--E--E--V--T--K--E--G--V--K--K--S--P-
9571 TCCCCAGGCTACCCACTGGTCTGTGTCACCCCCTGCGACCCTCATTTCCCCAGATAGGCT
3191 -S--P--G--Y--P--L--V--C--V--T--P--C--D--P--H--F--P--R--G--A-
9631 GTCATGAAGGAGCGCTGCAGTGAGGCCGGCATCAACCAGACATCTGTGCAGTTCAGCTGG
3211 -V--M--K--E--R--C--S--E--A--G--I--N--Q--T--S--V--Q--F--S--W-
9691 GAAGTGGCTGCCCCCACTGATGGCAATGGGGCCCGGTCTCCCTTTGAAACCATCACTGAC
3231 -E--V--A--A--P--T--D--G--N--G--A--R--S--P--F--E--T--I--T--D-
9751 AACACACCATTCACCAGTGTCAACCACATGGTCCTGGACAGCATTTACTTCAGCCGGGGG
3251 -N--T--P--F--T--S--V--N--H--M--V--L--D--S--I--Y--F--S--R--R-
Ex 64
9811 TTCCATGTGCGTTGTGTGGCCAAGGCTGTGGACAAGGTGGGCCATGTGGGGACCCCCTTA
3271 -F--H--V--R--C--V--A--K--A--V--D--K--V--G--H--V--G--T--P--L-
9871 AGGAGCAACATTGTTACCATTGGAACAGACAGTGCTATCTGCCACACACCAGTGGTGGCT
3291 -R--S--N--I--V--T--I--G--T--D--S--A--I--C--H--T--P--V--V--A-
9931 GGGACATCCAGAGGCTTCCAGGCTCAGTCCTTCATCGCAACCTTGAAATACCTGGATGTC
3311 -G--T--S--R--G--F--Q--A--Q--S--F--I--A--T--L--K--Y--L--D--V-
9991 AAACATAAGGAGCATCCGAACAGAATCCACATTTCGGTGCAGATCCCACACCAGGATGGA
3331 -K--H--K--E--H--P--N--R--I--H--I--S--V--Q--I--P--H--Q--D--G-
Ex 65
10051 ATGCTGCCCCTTATCTCCACCATGCCGTTGCACAACTTACATTTTCTACTGTCTGAGTCC
3351 -M--L--P--L--I--S--T--M--P--L--H--N--L--H--F--L--L--S--E--S-
10111 ATCTACAGACACCAGCACGTCTGCTCCAATTTAGTTACCACCATGACCTGAGAGGCATC
3371 -I--Y--R--H--Q--H--V--C--S--N--L--V--T--T--D--L--R--G--I-
10171 TCAGAGGCAGGGTTCCTGGATGATGTGGTCTATGATAGCACTGCCCTGGGGCCTGGCTAC
3391 -S--E--A--G--F--L--D--D--V--V--Y--D--S--T--A--L--G--P--G--Y-
Ex 66
10231 GATCGCCCCTTCCAGTTTGACCCCAGCGTGCGAGAGCCGAAGACCATCCAGCTCTACAAA
3411 -D--R--P--F--Q--F--D--P--S--V--R--E--P--K--T--I--Q--L--Y--K-
10291 CACCTGAACCTGAAGAGCTGCGTGTGGACCTTTGATGCTTATTATGACATGACTGAGCTG
3431 -H--L--N--L--K--S--C--V--W--T--F--D--A--Y--Y--D--M--T--E--L-
10351 ATTGACGTCTGTGGGGGCTCTGTAACGGCTGACTTCCAGGTGAGGGACTCTGCCCAGTCC
3451 -I--D--V--C--G--G--S--V--T--A--D--F--Q--V--R--D--S--A--Q--S-
Ex 67


10411 TTCTTGACAGTGCACGTGCCTCTATATGTGTCCTACATCTATGTGACAGCCCCCAGGGGC
3471 -F--L--T--V--H--V--P--L--Y--V--S--Y--I--Y--V--T--A--P--R--G-
10471 TGGGCCTCCTTGGAGCACCACACCGAGATGGAGTTTTCTTTCTTCTATGACACTGTTCTC
3491 -W--A--S--L--E--H--H--T--E--M--E--F--S--F--F--Y--D--T--V--L-
10531 TGGAGAACAGGAATCCAGACAGACAGCGTGCTCTCTGCAAGGCTTCAGATAATAAGAATC
3511 -W--R--T--G--I--Q--T--D--S--V--L--S--A--R--L--Q--I--I--R--I-
Ex 68
10591 TACATTCGAGAGGATGGCCGTCTTGTCATTGAATTCAAGACCCATGCCAAATTCAGAGGA
3531 -Y--I--R--E--D--G--R--L--V--I--E--F--K--T--H--A--K--F--R--G-
Ex 69
W R
10651 CAGTTTGTGATGGAGCATCACACTCTCCCAGAAGTGAAATCTTTCGTATTGACTCCAGAC
3551 -Q--F--V--M--E--H--H--T--L--P- -V--K--S--F- -L--T--P--D-
10711 CACCTAGGAGGAATTGAATTTGACTTGCAGCTATTATGGAGCGCTCAGACTTTTGATTCT
3571 -H--L--G--G--I--E--F--D--L--Q--L--L--W--S--A--Q--T--F--D--S-
10771 CCACATCAACTCTGGAGAGCCACAAGCTCTTATAACAGGAAGGACTACTCAGGAGAGTAC
3591 -P--H--Q--L--W--R--A--T--S--S--Y--N--R--K--D--Y--S--G--E--Y-
Ex 70
10831 ACCATCTACCTGATCCCTTGCACAGTGCAGCCCACACAGCCATGGGTTGACCCAGGAGAG
3611 -T--I--Y--L--I--P--C--T--V--Q--P--T--Q--P--W--V--D--P--G--E-
10891 AAGCCTTTGGCCTGCACTGCACATGCCCCAGAAAGATTCCTGATACCCATTGCATTCCAG
3631 -K--P--L--A--C--T--A--H--A--P--E--R--F--L--I--P--I--A--F--Q-
Ex 71
10951 CAGACCAACCGCCCTGTGCCAGTTGTGTATTCACTTAACACTGAATTTCAGCTCTGCAAT
3651 -Q--T--N--R--P--V--P--V--V--Y--S--L--N--T--E--F--Q--L--C--N-
S Y
11011 AATGAGAAGGTGTTCCTAATGGATCCAATACATCTGATATGTCATAGCAGAAATGGAT
3671 -N--E--K--V--F--L--M--D--P--N--T--S--D--M--S--L--A--E--M--D-
11071 TACAAAGGAGCCTTTTCAAAAGGTCAAATCCTTTATGGCCGAGTACTTTGGAATCCAGAA
3691 -Y--K--G--A--F--S--K--G--Q--I--L--Y--G--R--V--L--W--N--P--E-
Ex 72
11131 CAAAATCTTAATTCTGCTTACAAACTCCAGCTGGAGAAAGTCTATCTTTGTACGGGCAAG
3711 -Q--N--L--N--S--A--Y--K--L--Q--L--E--K--V--Y--L--C--T--G--K-
11191 GATGGTTATGTGCCTTTCTTTGATCCCACGGGGACAATCTACAATGAAGGGCCCCAGTAT
3731 -D--G--Y--V--P--F--F--D--P--T--G--T--I--Y--N--E--G--P--Q--Y-
11251 GGATGCATTCAGCCAAACAAACACCTAAAACACAGATTCCTGCTGTTGGACCGCAATCAG
3751 -G--C--I--Q--P--N--K--H--L--K--H--R--F--L--L--L--D--R--N--Q-
Ex 73
11311 CCAGAGGTAACTGATAAGTACTTCCATGATGTGCCTTTTGAGGCTCACTTTGCCTCTGAG
3771 -P--E--V--T--D--K--Y--F--H--D--V--P--F--E--A--H--F--A--S--E-
11371 TTGCCTGATTTCCATGTGGTCAGTAACATGCCAGGTGTGGATGGATTTACTCTAAAAGTA
3791 -L--P--D--F--H--V--V--S--N--M--P--G--V--D--G--F--T--L--K--V-
11431 GATGCACTCTATAAGGTGGAAGCA
3811 -D--A--L--Y--K--V--E--A--G--H--Q--W--Y--L--Q--V--I--Y--I--I-
Ex 74
11491 TCTCAGGGCCCCGGGTCCAGCGCTCTCTCACAGCTCCACTAGACGC
3831 -G--P--D--T--I--S--G--P--R--V--Q--R--S--L--T--A--P- --R--R-
11551 AACCGAAGGGACCTGGTAGAGCCCGATGGCCAGCTGATCCTTGATGATTCCCTCATCTAT
3851 -N--R--R--D--L--V--E--P--D--G--Q--L--I--L--D--D--S--L--I--Y-

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11611 GACAATGAAGGAGACCAAGTCAAGAATGGCACCAATATGAAGTCCCTGAATCTGGAGATG
 3871 -D--N--E--G--D--Q--V--K--N--G--T--N--M--K--S--L--N--L--E--M-

11671 CAAGAGTTGGCGGTAGCTGCGTCCCTGTCACAGACT[redacted]
 3891 -Q--E--L--A--V--A--A--S--L--S--Q--T-[redacted]

[redacted]ATCAACAGGAAATGC
[redacted]-I--N--R--K--C-

11791 CAGAAACAGAGGAAGAAGAAGCCCGCAGAGGACATTTTGGAAGAATATCCTCTGAATACC
 3931 -Q--K--Q--R--K--K--K--P--A--E--D--I--L--E--E--Y--P--L--N--T-

11851 AAGGTAGAAGTGCCCAAGAGGCACCCGGACCGGGTGGAGAAGAACGTGAATAGACACTAC
 3951 -K--V--E--V--P--K--R--H--P--D--R--V--E--K--N--V--N--R--H--Y-

11911 TGCACTGTGCGGAACGTCAACATCCTGAGTGAGCCTGAGGCGGCTTACACGTTCAAAGGT
 3971 -C--T--V--R--N--V--N--I--L--S--E--P--E--A--A--Y--T--F--K--G-

11971 GCTAAAGTCAAAAGACTGAATCTAGAAGTCAGAGTTCACAACAATTTACAAGATGGAACA
 3991 -A--K--V--K--R--L--N--L--E--V--R--V--H--N--N--L--Q--D--G--T-

12031 GAAGTTTAATGGAGGAGACCTATGTGTATTTTTTTCTAAAATCATTTTTATAAAATGGGG
 4011 -E--V--*-.....................................................
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Appendix 1: Human cDNA sequence for *FRAS1* and protein sequence of FRAS1: mutations highlighted in red, signal peptide in green, VWF domain in yellow, NG2 like domains in pink, CalXß domains in grey and transmembrane domain in turquoise.